Eesti Maaülikool
Estonian University of Life Sciences

RELATIONSHIPS BETWEEN BODY CONDITION, LIPOMOBILIZATION AND INSULIN RESISTANCE IN DAIRY COWS

TOITUMUSE, LIPOMOBILISATISOONI JA INSULIINIRESISTENTSUSE SEOSED PIIMALEHMADEL

PRIIT KARIS

A Thesis
for applying for the degree of Doctor of Philosophy
in Agricultural Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following three original publications (I–III). The articles are referred to in the text by their Roman numerals.

I Jaakson, H., **P. Karis**, K. Ling, A. Ilves-Luht, J. Samarütel, M. Henno, I. Jõudu, A. Waldmann, E. Reimann and P. Pärn, R. M. Bruckmaier, J. J. Gross, T. Kaart, M. Kass and M. Ots. 2018. Adipose tissue insulin receptor and glucose transporter 4 expression, and blood glucose and insulin responses during glucose tolerance tests in transition Holstein cows with different body condition. Journal of Dairy Science. 101:752-766.

II **Karis, P.**, H. Jaakson, K. Ling, R.M. Bruckmaier, J.J. Gross, P. Pärn, T. Kaart, and M. Ots. 2020. Body condition and insulin resistance interactions with periparturient gene expression in adipose tissue and lipid metabolism in dairy cows. Journal of Dairy Science. 103:3708–3718.

III **Karis, P.**, H. Jaakson, K. Ling, M. Runin, M. Henno, A. Waldmann and M. Ots. 2021. Body condition effects on dry matter intake and metabolic status during the transition period in Holstein dairy cows. Journal of Agricultural Science 32(1):49-58.

Contributions of authors to the publications, ordered alphabetically

Contribution	Publication		
	I	II	III
Idea and planning of the experiment	AW, HJ, IJ, KL, MH, MO, TK	HJ, KL, MO	AW, HJ, KL, MH, MO
Carrying out of the experiment, sample collection	AI, AW, HJ, JS, KL, MH, MK, MO, **PK**	HJ, KL, MO, **PK**	AW, HJ, KL, MO, MH, **PK**
Analysis of samples	AW, ER, HJ, JG, KL, MH, PP, RB	HJ, JG, KL, PP, RB	AW, HJ, KL, MH
Data analyses and visualization	**PK**, TK	**PK**, TK	**PK**
Data interpretation	HJ, KL, **PK**	HJ, KL, **PK**	HJ, KL, **PK**
Writing original draft	HJ	**PK**	**PK**
Manuscript review and editing	AW, HJ, JG, KL, MH, MK, MO, **PK**, RB, TK	HJ, JG, KL, MO, **PK**	AW, HJ, KL, MH, MO, MR, **PK**

AI - Aire Ilves-Luht, AW - Andres Waldmann, ER - Ene Reimann, IJ - Ivi Jõudu, JG - Joseph J. Gross, JS - Jaak Samarütel, HJ - Hanno Jaakson, MH - Merike Henno, MK - Marko Kass, MO - Meelis Ots, MR - Maksim Runin, **PK - Priit Karis**, PP - Pille Pärn, RB - Rupert M. Bruckmaier, TK - Tanel Kaart

ABBREVIATIONS

AST	–	Aspartate aminotransferase
AUC	–	Area under the curve
BC	–	Body condition
BCS	–	Body condition score
BHB	–	B-hydroxybutyrate
d	–	Day
DGAT2	–	Diacylglycerol O-acyltransferase 2
DM	–	Dry matter
DMI	–	Dry matter intake
ECM	–	Energy corrected milk
GAPDH	–	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	–	Glucose transporter member 4
INSR	–	Insulin receptor
IR	–	Insulin resistance
IVGTT	–	Intravenous glucose tolerance test
mRNA	–	Messenger ribonucleic acid
NEB	–	Negative energy balance
NEFA	–	Non-esterified fatty acids
LEP	–	Leptin
LIPE	–	Hormone sensitive lipase
LPL	–	Lipoprotein lipase
O	–	Optimal
OC	–	Over-conditioned
SAT	–	Subcutaneous adipose tissue
SLC2A4	–	Solute carrier family 2 member 4
SLC27A1	–	Solute carrier family 27 member 1
T	–	Thin
TMR	–	Total mixed ration

INTRODUCTION

Dairy farming has been and remains an important sector of the agriculture industry in Estonia. In the last century, the highest number of dairy cows was recorded before World War II, when over 400,000 cows were reared in Estonia (EPJ, 2021). The development of dairy cattle farming in Estonia was driven by the lucrative butter export market. For example, in 1931 Estonia exported 14.4 tons of butter, which made up 75% of the total agricultural products exported and 36% of the total export by value (Masso, 1932). The dairy cattle population decreased by half by the end of the war. In the Soviet era, it rose to just above 300,000 in the 1970s and remained more or less stable until the collapse of the regime. The turbulent times immediately after this caused a crisis in agriculture, and after five years of independence, only 190,000 cows remained, mainly at the expense of family farms. Today farms with a herd size over 300 cows produce 75% of total milk production. The number of animals is still declining and 84,300 dairy cows were reared in 2020. The decline in animal number has been in parallel with the increase in milk production per cow, annual production per cow has risen on average by 190 kg/cow per year over the last 20 years (EPJ, 2021). In 1990 1,200 thousand tons of milk were produced, while in 2018 800 thousand tons (Statistics Estonia, 2021). This means that with 70% fewer animals milk production has declined by only a third. Estonian cows in milk recording produced on average 10,400 kg of milk per lactation in 2020 (EPJ, 2021). Considering that in 2017, a cow in the USA produced over 35,000 kg of milk per year (Giebel, 2017), it seems that the physiological limit has not been reached and further increases in production per cow are expected. In order to support the demands on cows' metabolism by the ever-increasing milk production scientific knowledge on this topic must be concurrently enhanced.

The economic pressure causes selection bias toward cows of high genetic merit for milk production. These cows are able to partition more energy and nutrients into milk at the expense of the rest of the body. Every high-yielding dairy cow experiences a discrepancy in its energy balance (negative energy balance) at the beginning of lactation. The energy gained from feed is lower than her requirements and energy stored within her body is used. It is not out of the ordinary if a Holstein dairy cow loses 85 kg of her empty body weight during the first weeks

of lactation, which is equivalent to 564 kg of 4% fat-corrected milk (NRC, 2001).

The gain and loss of body fat reserves during lactation, or due to seasonal feed availability, must have been evident to farmers early on. The shortcomings of the use of body weight for the evaluation of adipose tissue relative volume was also known, namely the huge day-to-day mass variation and the differences of body sizes of cows (Kivimäe, 1939). Nevertheless, before the 1970s there were no good and easily applicable methods to assess the volume of adipose tissue in animals, according to Roche *et al.* (2013). A visual assessment of body adiposity was proposed in the 1970s, known as body condition scoring. Different protocols of body condition scoring were created, but that of Wildman *et al.* (1982) with a scale from 1-5, gained the most popularity. Their method was further improved and validated by Edmonson *et al.* (1989) and Ferguson *et al.* (1994).

At the end of the 1990s, the metabolic work group of the Chair (former department) of Animal Nutrition of the Estonian University of Life Sciences, led by senior research fellow Katri Ling, introduced and popularised the Edmonson *et al.* (1989) and Ferguson *et al.* (1994) body condition scoring methods in Estonia to evaluate negative energy balance, and organised scientific work on body condition score (BCS) applicability in cattle raised in Estonia (Ling *et al.*, 2000). This led to Jaak Samarütel's PhD thesis in 2009. He established the optimal body condition at calving for Estonian Holstein cows that supports maximum milk production concurrently with good metabolic health and fertility. In the 2000s, the metabolic work group mastered the technique of the intravenous glucose tolerance test (IVGTT) in order to assess insulin resistance (IR) in dairy cows (Jaakson *et al.*, 2007). This work resulted in the doctoral thesis of Hanno Jaakson (2012), who evaluated body condition effect on the development of IR in two Estonian dairy cattle breeds. The present thesis is a result of the Estonian University of Life Sciences Animal Nutrition chair's continuing work on this topic and combines BCS and IVGTT with a molecular approach.

1. REVIEW OF LITERATURE

1.1. Metabolic changes during the transition period

The transition period is typically defined as the six-week period starting from the third week before and leading up to the third week after calving (Drackley, 1999). Transition from pregnancy to lactation is associated with important readjustments in dairy cows' physiology and metabolism. These changes help the cow to adapt to lactation challenges and are vital for a successful transition (Gross and Bruckmaier, 2019). The different aspects of adaption to lactation in dairy cows have been extensively researched in recent decades. This has advanced our knowledge of metabolism, the control of dry matter intake (DMI), the role of adipose tissue and their interplay with immunity. Nevertheless, the transition period remains the most critical period in the production cycle of dairy cows as illustrated by high disease incidence and culling rates. Of 5,719 cows evaluated by Santos *et al.* (2010), 44% suffered clinical disease during the first two months of lactation.

In 1980 Baumann and Currie proposed a theory of homeorchesis. By definition, this means coordination of functioning of various tissues to support a physiological state, which during the transition period is either pregnancy before, or lactation after, calving. Neither of these highly energy-consuming states give any benefit to the dam. Nevertheless, nutrients are still partitioned toward the gravid uterus and mammary gland, even to a state where it may cause illness. This was elegantly demonstrated by Gross *et al.* (2011), who showed that if cows' energy intake was restricted in the second half of lactation cows adapted by lowering their milk production, and thereby their energy requirement. Whereas the same amount of energy deficit at the beginning of lactation did not cause a decrease in milk output, there was an increase in the mobilization of energy stored within the organism (Gross *et al.*, 2011).

Milk synthesis is a very energy and nutrient demanding process and determines the majority of the energy requirement of a lactating dairy cow. According to Estonian feeding requirements (Oll and Tölp, 1995), a cow weighing 600 kg needs approximately 110 MJ of metabolizable energy per day during the last week of gestation, whereas a fortnight later while producing 40 kg of energy corrected milk per day, it needs

275 MJ. The mammary gland uses 80% of this. Milk production of 40 kg is common for dairy cows within a week after calving. Milk production reaches its maximum about 60–90 days postpartum. This means that cows face a rapid increase in energy demand that is driven by the acceleration of milk synthesis.

To fulfil the energy requirement of 275 MJ to produce 40 kg of milk a cow has to eat approximately 24 kg of feed dry matter and the cow's digestive system has the volume to allow such DMI. Unfortunately, during the transition period several factors hinder feed intake. Calving activates the immune system and synthesis of pro-inflammatory cytokines increase in the last week before calving, causing immune-induced hypophagia (Kuhla, 2020; Brown and Bradford, 2021; Horst *et al.*, 2021). In addition, the increase in milk synthesis drives the increase in DMI and not vice versa; this means that intake always lags behind the increase in energy requirements. The nadir of DMI occurs on the day of calving at a time when the mammary gland is actively synthesising milk. Thus, dairy cows energy intake is always lower than her requirements at the beginning of lactation. This energy deficient period is termed negative energy balance (NEB) and it may last up to the 10^{th} lactation week (Bell, 1995). To compensate, energy stored within the organism, mainly in adipose tissue, is used. The loss of 50 kg of adipose tissue is common in dairy cows (Chilliard *et al.*, 2000).

Glucose and insulin concentrations in blood drop sharply at calving and remain low during the first weeks of lactation (Pires *et al.*, 2013) reflecting the shortfall in energy. At the same time, low glucose and insulin concentrations cause an increase in nutrient partitioning toward the mammary gland. Triglycerides from adipocyte lipid droplets are hydrolysed to glycerol and fatty acids. The latter are transported into blood, where they are known as non-esterified fatty acids (NEFA), to be oxidized for energy in various tissues, packed into lipoproteins in the liver or incorporated into milk fat (Drackley, 1999). Excessive flux of NEFA causes an increase in partial oxidation of NEFA in the liver that results in ketone bodies, mainly β-hydroxybutyrate (BHB) (Rojas-Morales *et al.*, 2016). The latter is transported into blood and can be used as an energy source by various tissues.

1.2. Adipose tissue

Adipose tissue is a connective tissue that is mainly composed of adipocytes, which are either white or brown. Brown adipocytes do not store the oxidation energy of fatty acids; instead, it is released as heat, which is vital for new-borns. Later in life the role and volume of brown adipocytes diminishes and in adulthood, more or less all of the adipocytes found in the body are white (Zoico *et al.*, 2019). In the lipid droplet of white adipocytes, mammals store excess energy in the form of triglycerides to be used when food is scarce. To carry out this function adipose tissue has a unique ability to greatly vary its volume. Lipids can make up 5% of the human athletes mass or 60% of the obese individuals mass (Lee *et al.*, 2013). Adipose tissue is stored in distinct places around the body, which are classified into five depots: subcutaneous, visceral, intramuscular, intermuscular, and bone marrow (Hausman *et al.*, 2014). The size of the depots are species-specific. In dairy cattle, the largest amounts of lipids are found intramuscularly (ca 50%), followed by visceral (30%), subcutaneous (15%), and others (5%) (Butler Hogg *et al.*, 1985; Cianzio *et al.*, 1985). In contrast, subcutaneous adipose tissue is the main depot in humans and pigs (Hausman *et al.*, 2014).

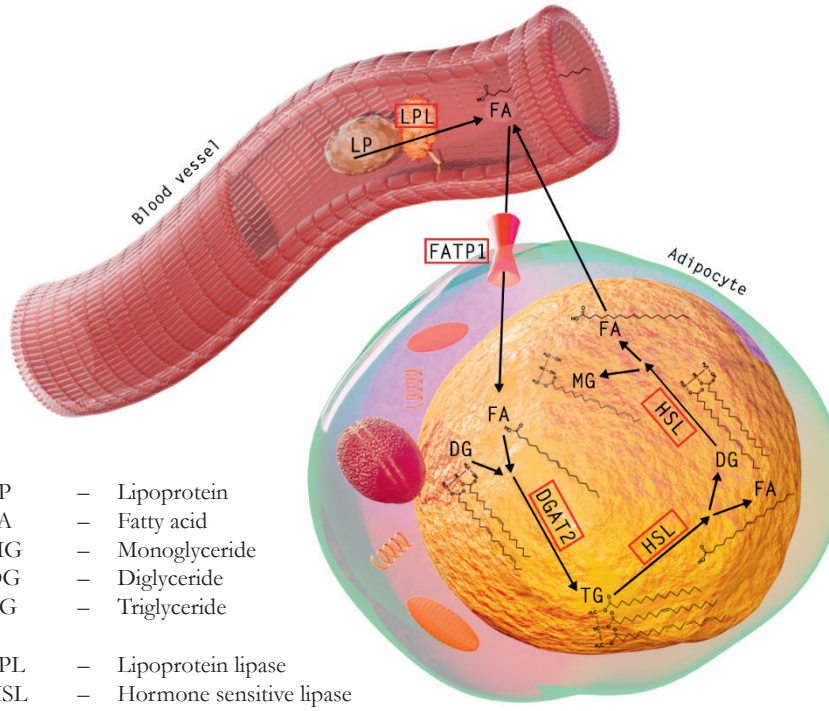
Historically steam-up diets during the dry period were used to increase the adipose tissue volume of dairy cows prior to calving (Friggens *et al.*, 2005). This strategy led to increased milk production during the following lactation. Currently it is understood that adequate adipose tissue volume is necessary to support milk production, but overconditioning of cows prior to calving is discouraged. Modern high-yielding dairy cows with too much adipose tissue mass (over-conditioned) at calving face a greater risk of high lipid mobilization that may lead to health problems; at the same time, under-conditioned cows produce less milk and have lower fertility (Roche *et al.*, 2009). This means that the amount of energy stored in adipose tissue affects metabolism and performance variables. For the farmer it is important to keep the cows in optimal condition, which supports milk production while concurrently lowering the risk of disease.

Adipose tissue is much more than just a passive tissue for energy storage. Zhang *et al.* (1994) discovered leptin, a cytokine – a small hormone-like protein – produced by adipose tissue. This discovery paved a path

for a new scientific discipline that has resulted in over 260 identified cytokines that are produced by adipose tissue (Lehr *et al.*, 2012) with either paracrine, endocrine or autocrine action. Adipose tissue is now considered the key tissue controlling energy metabolism (Polyzos *et al.*, 2016) and connecting metabolism and immunity (Contreras and Sordillo, 2011). The role of leptin has intrigued animal scientists due to its effect on voluntary feed intake. Increase in adipose tissue mass (Chilliard *et al.*, 2005) and adipocyte size (Depreeset *at al.* 2018) increase the production of leptin, which mediates an anorexigenic signal to the hypothalamus. Thus, leptin signals the repletion of body energy stores (Frühbeck, 2006). At the end of gestation, dairy cows reduce their DMI (Grummer *et al.*, 2004). This drop is most severe in the last week of gestation, and the cow eats the least on the day of calving (Ingvartsen and Andersin, 2000). Overconditioning and overfeeding in the dry period cause an increase in blood leptin concentration, which might then induce the DMI drop (Kokkonen *et al.*, 2005; Locher *et al.*, 2015). At the same time, cows that eat well have a better chance to avoid ill health during the transition period (De Koster *et al.*, 2019; Cardoso *et al.*, 2020). On the other hand, increasing evidence indicates an immune function association with the DMI drop. Pro-inflammatory cytokines mediate anorexigenic signals to the hypothalamus (Kuhla, 2020). During the transition period, the adipose tissue is remodelled to support intense lipolysis. This includes infiltration of immune cells into adipose tissue and increased production of pro-inflammatory cytokines (e.g. tumour necrosis factor α, interleukin 6) (Contreras *et al.*, 2017). This causes a reduction of insulin signalling, increase in lipolysis and a systemic inflammatory state (Contreras *et al.*, 2018).

In adipose tissue, there are simultaneous processes of lipogenesis, storage of fats, and lipolysis, the hydrolysis of stored fats. The balance between these processes determines the net gain or loss of adipose tissue volume. Amongst lipogenic factors, lipoprotein lipase (LPL) is an enzyme encoded and translated by adipocytes, but is transported into a nearby capillary, where it hydrolyses triglycerides from lipoproteins and thus determines the availability of fatty acids for adipocytes (Kersten, 2014). Long-chain fatty acid transport protein 1 (FATP1, the protein of gene *SLC27A1*) localizes in cell membranes and forms a complex with protein CD36 and facilitates the transport of fatty acids into cell cytoplasm (Wu, 2018). Diacylglycerol O-acyltransferase 2 (DGAT2) catalyses the formation of triglyceride from diglyceride, whereas the

lipolytic enzyme hormone sensitive lipase (HSL, the protein of gene *LIPE*) does the opposite, hydrolysing triglyceride to diglyceride (Wu, 2018). The abundance and activity of these key proteins determine the balance between lipogenesis and lipolysis (Figure 1).



LP – Lipoprotein
FA – Fatty acid
MG – Monoglyceride
DG – Diglyceride
TG – Triglyceride

LPL – Lipoprotein lipase
HSL – Hormone sensitive lipase
FATP1 – Long-chain fatty acid transport protein 1
DGAT2 – Diacylglycerol O-acyltransferase 2

Figure 1. A simplified mode of action of insulin on lipid metabolism in the adipocyte.

1.3. Body condition score

Body condition score (BCS) is a good proxy for the estimation of total amount of fat reserves, or more specifically, for the volume of subcutaneous adipose tissue (SAT). It is an easy and quick method applicable in field conditions. Several BCS protocols and scales have been developed, e.g 0–5, 1–5, 1–8, 1–10 (Roche *et al.*, 2009), but the general idea is the same in all of these. By visual assessment and palpation of certain areas of the body, usually pelvis, lumbus and tail head, a score is given. A lower score indicates emaciation and higher score obesity. The most commonly used scale of 1–5 by Edmonson *et al.* (1989) is used in this thesis whenever BCS is mentioned. Although the assessment might

seem subjective, the scores given by different assessors are comparable (Edmonson *et al.*, 1989; Ferguson *et al.*, 1994; Samarütel *et al.*, 2001). It describes very well the volume of subcutaneous adipose tissue, r^2=0.74–0.93, (Roche *et al.*, 2013), and it correlates well with the adipocyte volume in different adipose depots, r > 0.9 (De Koster *et al.*, 2016b).

The BCS method has advantages over the weighing of the animal. First, weighing is very labour-intensive. Secondly, the fat-free mass of cows is different; a cow with small body size has relatively more adipose tissue compared to a cow that has an equal weight, but a large body size. Thirdly, the increasing mass of the foetus at the end of gestation, or the increasing DMI at the beginning of lactation, overshadows the actual loss of adipose tissue. Feed particles stay in the digestive tract for approximately 45 hours (Pond *et al.*, 2005). It is estimated that a 1 kg increase in DMI will increase the fill by 2.5 to 4 kg (NRC, 2001).

However, there are also some drawbacks to the BCS method. It is assumed that if a cow loses or gains adipose tissue mass it does so proportionally in all of the adipose tissue depots, but this assumption is not valid if the change is rapid, thus, it might lack the sensitivity over the short term to detect changes in internal fat depots (Drackley *et al.*, 2014). In addition, the adipose tissue depots are not in fact uniform as they differ in their endocrine activity (Depreester *et al.*, 2018), enzyme activity (Locher *et al.*, 2011; Ruda *et al.*, 2019), and adaption to lactational challenges (Kenez *et al.*, 2019). Visceral adipose tissue, located in the abdominal cavity, is a metabolically more active tissue compared to SAT. It produces more cytokines and cows lose proportionally more fat from that depot at the beginning of lactation. In addition, visceral adipose tissue drains directly into the liver through the hepatic portal vein, thus it has a higher potential to overload the liver with fats. BCS also poorly describes the largest fat depot in the dairy cow, the inter- and intramuscular fat (r^2=0.43) (Roche *et al.*, 2013).

Prior research on Estonian Holstein dairy cows has led to a classification of BCS ranges as follows: thin (BCS ≤ 3.0), optimal (BCS = 3.25–3.5), over-conditioned (BCS ≥ 3.75) (Samarütel, 2009). The optimal body condition range is still debated and may depend on the genotype, as cows with higher genetic merit of milk production are prone to higher rates of lipolysis compared to their lesser productive peers (Khan *et al.*, 2013).

1.4. Distinct features of glucose and insulin metabolism in ruminants

Insulin is a hormone secreted by pancreatic β-cells and its production and release is mainly regulated by the glucose concentration in blood (Wu, 2018). In general, insulin activates pathways responsible for energy storage within the body, e.g. glucose uptake, lipogenesis and glycogenesis (Bell and Bauman, 1997). In addition, insulin is a potent inhibitor of lipolysis; its signalling reduces the cellular concentration of cyclic adenosine monophosphate (cAMP) that in turn reduces protein kinase A (PKA) mediated lipolysis (Bolsoni-Lopes and Alonso-Vale, 2015).

Insulin is mostly known for its effect on facilitating glucose flux from blood into cells. As show in Figure 2, upon coupling with the insulin receptor (INSR) on the cell membrane of insulin-sensitive tissues, mainly muscle and adipose tissue, the downstream signalling causes translocation and fusion of glucose transporter member 4 (GLUT4) with the cell membrane. The latter protein facilitates glucose transport into cells. Therefore, INSR and GLUT4 represent respectively the start and end of insulin signalling responsible for facilitated cellular glucose uptake (Lewis *et al.*, 2002). In addition to facilitating glucose uptake insulin, and tissues sensitivity to insulin, play a central role in the adjustment of energy partitioning between tissues and in balancing lipogenesis and lipolysis (Bell and Bauman, 1997).

A distinct feature of ruminant animals is pre-gastric fermentation of feed by microorganisms. Starch is enzymatically hydrolysed to glucose in monogastric animals, whereas in ruminants it is fermented into volatile fatty acids in the rumen. Dairy cows are grazers and their diet in the wild would be low in starch. This means that negligible amounts of starch reach the small intestine of ruminants, and their digestive system has evolved so that the activity of enzymes involved in starch digestion is low and glucose absorption is poor (Harmon and Swanson, 2020). Modern farming has introduced concentrates into the diets of dairy cows which has increased the amount of glucose absorbed from the intestine, but dairy cows still heavily rely on hepatic glucose production (gluconeogenesis) (Aschenbach *et al.*, 2010).

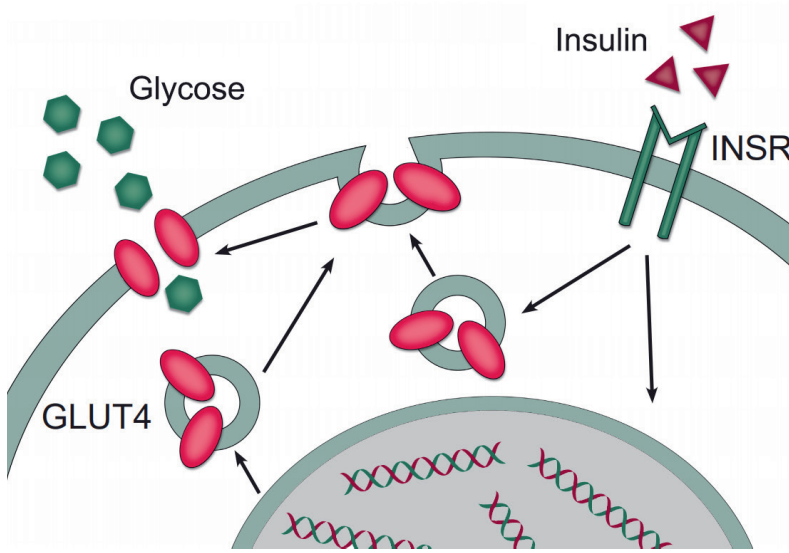


Figure 2. A simplified mode of action of insulin on glucose metabolism. Insulin signalling starts with its binding to insulin receptor (INSR). The downstream signalling causes the increased gene expression and protein synthesis of glucose transporter member 4 (GLUT4) and its fusion with the cell membrane. The latter protein transports glucose into the cell.

In the basal state of nonpregnant, nonlactating humans and ruminants, insulin mediates approximately 20% of glucose disappearance from blood (Baron *et al.*, 1988; Patterson *et al.*, 1993), half of which is transported into muscle tissue (Baron *et al.*, 1988). In experimentally induced hyperinsulinaemic condition, insulin-mediated glucose disappearance can be as high as 95% in humans, but about 70% in ruminants (Patterson *et al.*, 1993). This is caused by the difference in insulin responsiveness of the two species. Insulin responsiveness is defined as the maximal effect of insulin on target tissue and is measured as glucose disappearance. The maximal glucose disappearance is approximately 10 mg/kg min in humans (Bergman *et al.,* 1985), but 3.3 mg/kg min in sheep (Petterson *et al.,* 1993). Insulin sensitivity, defined as the insulin concentration to cause a half-maximal response, is comparable between humans, sheep and dairy cows (Bergman *et al.,* 1989; Patterson *et al.,* 1993; De Koster *et al.,* 2015).

The growth of the gravid uterus and milk synthesis are glucose demanding processes. Insulin does not control the uptake of glucose by the uterus or the udder. Glucose flux, and therefore the growth of the foetus and milk synthesis, can continue even when feed is scarce

and glucose concentrations, as well as those of insulin, are low. In the last weeks preceding parturition the glucose requirement of the gravid uterus is approximately 1 kg per day (De Koster and Opsomer, 2013). The glucose requirement of the udder to produce 45 kg of milk is approximately 3.2 kg (Kronfeld, 1982). Throughout the production cycle, dairy cows are either pregnant, lactating, or both at the same time. It is estimated that insulin mediates about 8% of glucose disappearance in lactating (Rose *et al.*, 1997) and 15% in non-lactating pregnant cows (Petterson *et al.*, 1993) in basal conditions. In hyperinsulinaemia the corresponding values are 39% (Rose *et al.*, 1997) and 52% (Patterson *et al.*, 1993).

1.5. Insulin resistance

Insulin resistance (IR) is defined as whenever a normal concentration of insulin produces a less than normal biological response. Insulin signalling can be subdivided into sensitivity, the maximal effect of insulin on target tissue, and responsiveness, the required concentration of insulin to elicit half-maximal effect (Kahn, 1978). IR can manifest in a decrease in sensitivity, decrease in responsiveness or both at the same time. This situation of insulin resistance is deemed to develop during the dry period (De Koster and Opsomer, 2013). This condition is similar to the IR described in humans that precedes type 2 diabetes mellitus and stems from adipose tissue dysfunction (Johnson and Olefsky, 2013). The main manifestation of IR in humans is the high glucose concentration in blood, whereas in cows, the excess glucose is synthesised into lactose and IR's effect on lipid metabolism is more important. This is especially evident during the transition period. In that period cows' endocrine status favours lipid mobilization, mainly through adrenergic signalling, and insulin is its main antagonist (Contreras *et al.*, 2018). Therefore low insulin synthesis or a blockage in its signal transduction in adipose tissue causes intensified lipolysis that may result in lipotoxicity due to the high concentration of NEFA in blood. Zachut *et al.* (2013) and Zachut and Moallem (2017) have confirmed that cows that lose more body mass postpartum were in a more pronounced IR state prepartum. A high concentration of NEFA in blood is a risk factor for an array of production diseases during transition period, e.g. fatty liver and ketosis (Ospina *et al.* 2010). In addition, NEFA reduces the activity of immune cells and thereby also makes the host vulnerable to infectious diseases. High lipolysis causes immune cells infiltration into adipose tissue and

systemic inflammation that in turn increases lipolysis (Contreras *et al.*, 2017).

The gold standard for the measurement of insulin signalling is hyperinsulineamic euglycaemic clamp test that was developed by DeFronzo *et al.* (1979). In dairy cows, this method is carried out by catheterization of both jugular veins. Insulin is continuously infused into one catheter at a beforehand-decided rate. Glucose is infused into the other vein and the concentration in blood is continuously monitored to maintain basal level in blood (euglycaemia). If the insulin concertation is high enough, then hepatic glucose production is turned off and the total glucose utilization of the animal is equal to the infusion rate. With the addition of several insulin infusion rates and labelled glucose, insulin response curves and hepatic glucose production can be calculated. However, this method is very invasive, labour-intensive, time-consuming and expensive, so it is only suitable if a small number of animals are enrolled and it cannot be performed in field conditions (De Koster and Opsomer, 2013).

The glucose tolerance test, even though still invasive in nature, is a more suitable method to measure IR in a large number of animals. According to this method, a single glucose dose is administered either orally or intravenously. The former is the method used to estimate IR in humans and the latter, called the intravenous glucose tolerance test (IVGTT), is used in animals. The metabolic response caused by the glucose dose is monitored and blood samples are taken at regular intervals up to 120 minutes after administration. The downside of this method is that it cannot differentiate between the effects of insulin sensitivity, insulin responsiveness, hepatic glucose production or β-cell insulin production. Nevertheless, in dairy cows this method correlates well with hyperinsulinaemic euglycaemic clamp test results (De Koster *et al.,* 2016).

In addition, several surrogate indices have been developed in human studies to assess IR status, e.g. RQUICKI and HOMA-IR. They rely on the measurement of the concentration of certain metabolites (e.g. insulin, glucose, BHB, NEFA) in one blood sample. These indices have been used for veterinary use, but they are not precise enough and should not be used to assess IR status in dairy cows (Saed Samii *et al.*, 2019; De Koster *et al.,* 2016a).

There are few studies integrating BCS, IVGTT, and adipose tissue gene expression at the mRNA and protein levels. A clear understanding of cause-and-effect relationships between the IR and the physiological and metabolic status of cows, as well as understanding of the molecular mechanisms of insulin signalling are still lacking. This thesis tries to advance the state-of-the-art knowledge in those subjects.

2. AIMS OF THE STUDY

The main hypothesis of the thesis was that adipose tissue volume prepartum and lipolysis postpartum are both related to whole-body IR and to gene expression in adipose tissue.

Therefore, the aims were

1. to examine the effect of BCS in Holstein dairy cows during the dry period on:
   - longitudinal dynamics of DMI, BCS, blood metabolite concentrations and milk composition around calving,
   - the dynamics of glucose, insulin and NEFA following intravenous glucose infusion on 21 days before (d –21) and 21 days after calving (d 21) to assess differences in IR status,
   - concentrations of glucose metabolism-related proteins INSR and GLUT4, and expression of corresponding mRNAs *INSR* and *SLC2A4* in SAT on d –21 and d 21,
   - expressions of lipid metabolism-related genes *SLC27A1, LEP, LPL, DGAT2, LIPE* in SAT on d –21 and d 21.
2. to investigate relationships between IR status with gene expression in SAT and lipid mobilization around parturition and to integrate all of the results for a better understanding of transition cow metabolism.

3. MATERIAL AND METHODS

3.1. Experimental design (I, II, III)

The European Council Directive regarding the protection of animals (Council directive 2010/63/EU) and the Estonian Animal Protection Act (Animal Protection Act, 2000) have been complied with in this study. In addition, the study has been approved by the Committee for Conducting Animal Experiments at the Estonian Ministry of Rural Affair. The study was carried out over two consecutive years (Jan 2013 – Feb 2015) on the experimental farm of the Estonian University of Life Sciences with a herd size of 120 cows and a mean annual milk yield of 9,200 kg/cow at the time of the experiment. Cows BC were assessed fortnightly according to the method described by Edmonson *et al.* (1989) starting from dry-off, at approximately 60 days before expected calving. Cows whose BCS 28 days before expected calving (d –28) was the same as at dry-off were eligible for the study. In total, 46 Estonian Holstein cows were assigned into three experimental groups on the basis of BCS on d –28 as follows: BCS ≤ 3.0 [2.25–3.00, Thin (T)]; BCS 3.25–3.5 [Optimal (O)]; BCS ≥ 3.75 [3.75–4.50, Over-conditioned (OC)]. Due to culling in the first two weeks of lactation, four cows were excluded from the study. The remaining 42 cows were equally distributed between three experimental groups, 14 in each. Cows were enrolled in accordance with a blocked design with each block consisting of three cows, one from each experimental group. Fortnightly BCS assessment continued until d 42 postpartum. On d –28 cows were removed from the dry cow barn to tie-stall housing and on d 3 moved to a free-stall barn with a milking parlour.

One cow from group T was culled before the final blood sample due to udder disease. In addition, two cows from group T and three cows from group OC were culled before the end of lactation. The culling reasons were two incidences of hoof disease and three incidences of udder disease.

3.2. Feeds and diets (I, II, III)

The feeding data given in Table 1 represent means for the whole two-year study period. Silage samples were taken twice weekly. Other feeds were sampled on a batch basis. All samples were analyzed for dry matter

Table 1. Ingredients and chemical composition (Mean ± SD) of total mixed rations diets.

	Diets				
Item	Far-off Until d -15	Close-up d -14 to d -1	Lactation 1 d 1 to d 6	Lactation 2 d 7 to d 14	Lactation 3 from d 15
Ingredient (g/kg of DM)					
Grass silage	955 ± 81	599 ± 71	604 ± 72	460 ± 49	384 ± 37
Hay	33.6 ± 81	28.3 ± 69	28.6 ± 70	20.3 ± 49	15.6 ± 37
Barley meal	-	301 ± 10	303 ± 10	309 ± 0.1	296 ± 0.2
Corn meal	-	-	-	64.3 ± 0.0	120 ± 0.1
Rapeseed cake	-	47.1 ± 0.6	47.5 ± 0.6	129 ± 0.0	168 ± 0.1
Mineral-vitamin feed	11.7 ± 0.1[1]	7.82 ± 0.2[1]	10.5 ± 0.3[2]	8.58 ± 0.0[2]	7.99 ± 0.0[2]
Anionic mineral feed	-	10.4 ± 0.2[3]	-	-	-
Limestone	-	6.26 ± 0.2	-	4.29 ± 0.0	4.41 ± 0.7
Sodium chloride	-	-	5.78 ± 0.1	4.72 ± 0.0	4.40 ± 0.0
DM of diet	348 ± 78	435 ± 64	433 ± 64	483 ± 53	511 ± 45
Chemical composition (g/kg of DM)					
Crude protein	131 ± 10.5	144 ± 7.2	145 ± 7.3	161 ± 5.6	169 ± 4.8
Metabolizable protein	72.8 ± 3.3	86.6 ± 2.1	87.3 ± 2.1	97.9 ± 1.6	104 ± 1.3
Metabolizable energy	8.70 ± 0.3	10.1 ± 0.2	10.2 ± 0.2	10.9 ± 0.1	11.3 ± 0.1
Neutral detergent fiber	534 ± 51	455 ± 32	458 ± 32	410 ± 25	380 ± 22
Acid detergent fiber	387 ± 47	278 ± 29	280 ± 29	242 ± 22	220 ± 18
Calcium	11.4 ± 1.9	10.3 ± 1.3	9.33 ± 1.3	9.54 ± 1.0	8.97 ± 0.8
Phosphorus	3.42 ± 0.4	4.06 ± 0.2	4.03 ± 0.2	4.48 ± 0.2	4.77 ± 0.2

[1] Composition (as-fed basis): 170 g/kg of Ca; 50 g/kg of P; 30 g/kg of Na; 140 g/kg of Mg; 30 g/kg of S; 1000 mg/kg of Cu; 4500 mg/kg of Zn; 4000 mg/kg of Mn; 40 mg/kg of Se; 50 mg of Co; 200 mg of I; 800000 IU/kg of vitamin A; 190000 IU/kg of vitamin D; and 8000 IU/kg of vitamin E.

[2] Composition (as-fed basis): 150 g/kg of Ca; 35 g/kg of P; 75 g/kg of Na; 90 g/kg of Mg; 1 g/kg of S; 4000 mg/kg of Cu; 6667 mg/kg of Zn; 6452 mg/kg of Mn; 94 mg/kg of Se; 109 mg of Co; 650000 IU/kg of vitamin A; 150000 IU/kg of vitamin D; and 4000 IU/kg of vitamin E.

[3] Composition (as-fed basis): 9 g/kg of Ca; 1 g/kg of P; 5 g/kg of Na; 100 g/kg of Mg; 1000 mg/kg of Cu; 5000 mg/kg of Zn; 2000 mg/kg of Mn; 27 mg/kg of Se; 40 mg of Co; 100 mg of I; 1000000 IU/kg of vitamin A; 60000 IU/kg of vitamin D; and 10000 mg/kg of vitamin E, 100000 μg/kg of biotin.

(DM), while chemical composition was analyzed using methods approved by the AOAC (2005) and nutritive value was calculated once weekly for silage samples, or on a batch basis for other feeds. If necessary, the proportions of the rations' ingredients were corrected to meet required composition and nutritive values.

Cows were fed a total mixed ration (TMR) *ad libitum* twice daily, at 05.30 and 14.30 h. Ingredients and chemical composition of TMR are presented in Table 1. Diets were based on grass-clover silages that were supplemented with differing amounts of concentrates, vitamins, and minerals according to lactation stage to fulfil Estonian feeding recommendations (Oll and Tölp, 1995; Kärt *et al.,* 2002). Between d –28 to d 2 cows were fed individually and orts were collected and weighed before fresh feed was offered. Daily dry matter intake (DMI) was calculated in this period by subtracting the weight of orts from that of the feed offered. To calculate the prepartum DMI drop average DMI between d –14 to –8 were calculated for each cow, which was thereafter subtracted from DMI on d –1.

3.3. Milk samples and analyses (I, III)

The cows were milked twice daily at 5.00 and 15.00 h. Milking parlour hardware (DeLaval, Tumba, Sweden) recorded milk yields. Milk samples were taken twice per week until the end of lactation. Samples were stabilized with bronopol (Broad Spectrum Microtabs, D&F Control Systems Inc., Norwood, MA, USA) and analysed for fat and protein with an automatic infrared milk analyzer (System FT+, Foss Electric, Hillerød, Denmark) in the Milk Analysis Laboratory of Estonian Livestock Performance Recording Ltd. ECM-yields were calculated according to Sjaunja *et al.* (1991). A fourth order polynomial was fitted to milk, ECM, milk fat and milk protein production values. The area under the curve was calculated for each of the variables as the definite integral of the fitted polynomial and used as an estimate for total production between 42 and 305 days in milk.

3.4. Weekly blood samples and analyses (I, II, III)

Blood samples were taken on d –21 ± 2.3, d –14 ± 2.1, d –7 ± 2.6, d 7 ± 0.9, d 14 ± 0.8, d 21 ± 1.0, d 28 ± 1.1 and d 42 ± 2.1 at around 10.00 h from the coccygeal vein into vacuum tubes containing Li-heparin (VACUETTE®, Greiner Bio-One International GmbH, Kremsmünster, Austria). Samples were centrifuged (5,000 × g, 15 min, +4 °C) and stored at –80 °C. A Clinical chemistry analyzer (ERBA XL300, ERBA Diagnostics Mannheim GmbH, Mannheim, Germany) was used to measure the concentration of non-esterified fatty acids (NEFA) (Cat. No. FA 115; Randox Laboratories Ltd., Crumlin, United Kingdom), β-hydroxybutyrate (BHB) (Cat. No. RB 1007; Randox Laboratories Ltd.), aspartate aminotransferase (AST) (product code XSYS016, ERBA Diagnostics Mannheim GmbH) and glucose (product code XSYS012, ERBA Diagnostics Mannheim GmbH). All of the inter- and intra-assay coefficients of variation were below 8.3% and 6.1%, respectively.

Insulin from weekly blood samples was analysed by bovine-optimized sandwich ELISA (Cat. No. 10-1201-01; Mercodia AB, Uppsala, Sweden), with a detection limit of 0.025 ng/mL, on a microplate reader (SunriseTM, Tecan Group Ltd., Switzerland); results were calculated using cubic spline regression (Magellan™ data analysis software; Tecan Group Ltd.). The inter-assay coefficients of variation for plasma insulin concentrations of 0.2 ng/mL and 1.6 ng/mL were 4.6% and 6.6%, respectively and the intra-assay coefficients of variation were 4.2% and 4.1%, respectively.

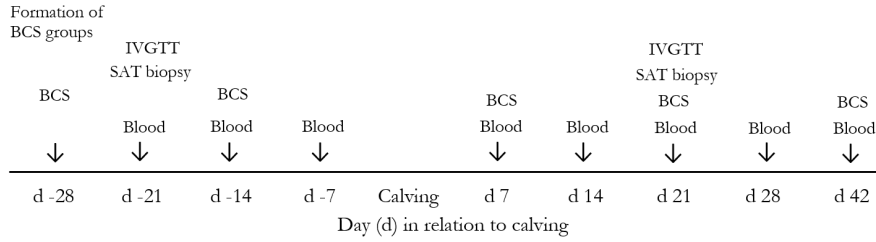


Figure 3. A timeline of blood sampling, body condition score (BCS) assessing, subcutaneous adipose tissue (SAT) biopsies and intravenous glucose tolerance test (IVGTT).

Insulin from IVGTT blood samples were analysed using radioimmunoassay as described by Vicari *et al.* (2008). The intra- and inter-assay coefficients of variation for insulin were both < 10%.

3.5. Intravenous glucose tolerance test (I, II)

The IVGTT was carried out on d –21 and on d 21. Relative to the start of jugular-vein infusion of a 0.15 g/kg BW glucose (40% solution), blood samples were collected at –5 (basal), 5, 10, 20, 30, 40, 50 and 60 min. Blood samples were centrifuged and plasma stored at –80 °C until analysed for glucose, insulin and NEFA as described above.

To describe glucose and insulin responses to the IVGTT increment (mg/dL) and area under the curve 5–60 min (mg/dL × min) were calculated. The increment was defined as the difference between maximal and basal concentration during IVGTT. The area under the curve was calculated as the definite integral of the fifth-order polynomial fitted to values corrected for basal concentration.

NEFA decrease and latency were calculated. The decrease was calculated as the difference between basal and minimum concentrations and expressed as a percentage of the basal concentration. Latency was defined as the point where the second order polynomial fitted NEFA values between minutes 5–60, expressed as the percentage of basal NEFA, crossed the x-axis. If the value was lower than the average infusion time of 2 min, then the latency period length was presumed to be 2 min.

3.6. Adipose tissue biopsies (I, II)

SAT biopsies were taken after blood sampling for metabolic profiling and prior to the procedures of IVGTT from the pin bone region of each cow after local anaesthesia (Lidocaine – Grindex 20 mg/mL injection solution) and under aseptic conditions on d –21 and d 21. A 2-cm skin incision was made and a tissue sample of about 3 g was collected, flushed with physiological saline, mounted into screw-cup tubes (Axygen®, Corning Incorporated, 767 Fifth Avenue, NY US), immediately frozen in liquid nitrogen and stored at –80 °C

3.7. Measuring gene expression (I, II)

For RNA analyses AT was homogenized using the FastPrep®-24 (116004500 MP BIOMEDICALS, France) with a Metal Bead Lysing Matrix (Cat. No. 6925-050) in QIAzol Lysis Reagent (Cat. No. 79306,

Qiagen GmbH, Hilden, Germany), and total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Cat. No. 74804, Qiagen GmbH). RNA integrity (RIN) was determined using a 2100 Bioanalyzer (Agilent Technologies, Massy, France) and RNA 6000 Nano kit (Agilent Technologies Inc., Santa Clara, CA, USA) and ranged from 6.7 to 8.0. RNA concentrations were determined with the Qubit® RNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total RNA was stored at –80 °C until cDNA synthesis using the QuantiTect Reverse Transcription Kit (Cat. No. 205311, Qiagen GmbH). Synthesized cDNA was stored at –20 °C until used for PCR.

Multiplex quantitative real-time PCR was carried out on one 384-well plate for each target gene together with the reference gene in four replicates using the TaqMan® Gene Expression Master Mix (Cat. No. 4369016, Thermo Fisher Scientific Inc.) with the ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA). Primers and probes were from Thermo Fisher Scientific Inc. (TaqMan® Gene Expression Assays, Thermo Fisher Scientific Inc., NY, USA). Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* (Assay ID: Bt03210913_g1) was used as a reference gene for normalization of mRNA expression levels of insulin receptor (*INSR*; forward primer sequence: TCCTCAAGGAGCTGGAGGAGT, reverse primer sequence: GCTGCTGTCACATTCCCCA, probe sequence: ATCATCTTCAGGCCCTGTTGCCGAG), solute carrier family 2 member 4 (glucose transporter member 4) (*SLC2A4*; Assay ID: Bt03215323_m1), leptin (*LEP*; Assay ID: Bt03211909_m1), lipoprotein lipase (*LPL*; Assay ID: Bt03240499_m1), solute carrier family 27 member 1 (fatty acid transport protein 1) (*SLC27A1*; Assay ID: Bt03252013_m1), diacylglycerol O-acyltransferase 2 (*DGAT2*; Assay ID: Bt03259839_m1) and hormone sensitive lipase (*LIPE*; Assay ID: Bt03253691_m1) genes. Cycle threshold (Ct) values were used to calculate the relative gene expression level using the ΔCt method. For statistical analysis the mean of 4-replicates' of Ct values were used.

3.8. Measuring protein concentration (I)

For the measurement of INSR and GLUT4 approximately 20–200 mg of adipose tissue was homogenized using the FastPrep-24 (116004500 MP BIOMEDICALS, France) and Lysing Matrix D 2 mL tubes (Cat. No. 116913100, MP Biomedicals, LLC, 3 Hutton Center Drive, Suite

100, Santa Ana, CA, USA). Prior to homogenizing, the tissue was washed in 1 mL PBS and transferred to a homogenizing tube containing 1 mL of homogenizing solution. The homogenizing solution was based on *phosphate-buffered* saline (PBS) with a proteinase inhibitor (Complete Ultra Tablets, Cat. No. 05892791001, Roche Diagnostics, IN, USA). The FastPrep-24 Instrument program was 2×30 min with a speed of 6.5 m/s. Dry ice was used for cooling during homogenization. The homogenate was transferred into a new tube and centrifuged at room temperature at 1,000 × g for 10 min. The clear homogenate below the upper fat layer was transferred into a new tube and used for analyses. The total protein concentration was measured using the Modified Lowry Protein Assay Kit (Cat. No. 23240, Thermo Fischer Scientific Inc, 168 Third Avenue. Waltham, MA, USA) according to the manufacturer's protocol in three replicates. Amounts of 50–150 μg of total protein were used for each ELISA reaction. The concentrations of INSR and GLUT4 were measured in two replicates and their means were used for data analysis. For ELISA, commercial kits were used according to the manufacturer's protocol (INSR: Cat. No. E11A0445, GLUT4: Cat. No. E11G0201, antibodies-online Inc., GA, USA).

3.9. Statistical analysis (I, II, III)

Before statistical analysis the relative mRNA concentrations of *INSR* were multiplied by 1,000*; SLC2A4, LEP* and *SLC27A1* were multiplied by 100; *LIPE*, *LPL* and *DGAT2* by 10 to avoid negative values after logarithmic transformation. Statistical analyses were performed with the software R, versions 3.3.1 (I, II) and 3.5.0 (III). To estimate the effect of BCS group and time on the dependent variables a mixed linear model was fitted. Pre- and postpartum IVGTT data and blood sample data were modelled separately. Modelling was performed with the function "lmer" in the package "lme4" the least square means (LSM, alias marginal or model based means) were estimated with the function "lsmeans" for study I, and with function „emmeans" for studies II, III. Pairwise comparison of LSM was performed with the function "contrast". P-values were adjusted for multiple testing with the Tukey method. The significance of the effect of factors considered in the model was estimated with the type 2 Wald chisquare test with the function "Anova". Two analyses were performed for the variables that were not normally distributed: initially the models were fitted and the LSM were estimated on an arithmetic scale and subsequently the P-values were estimated fitting the same models

on logarithm-transformed values. Correlations and regression lines were calculated with the function "ggscatter" of the package "ggpubr". In the calculation of Pearson correlations and regression, the BCS groups were not differentiated. Statistical significance was declared at $P \leq 0.05$.

4. RESULTS

4.1. DMI, milk and BCS

DMI from d –14 to d –8 was lower in OC cows compared to T cows ($P < 0.05$; Table 2). In addition, OC cows' DMI were lower than those of T cows on d –3 and d –1 ($P < 0.05$; Figure 4). On d 1 and 2 postpartum DMI was the greatest in T cows ($P < 0.01$). A DMI drop was significant for all groups (T – $P < 0.05$; O and OC – $P < 0.01$), but it did not differ between groups.

Table 2. Milk yield and composition, dry matter intake (DMI), and body condition score (BCS) characteristics in multiparous Holstein cows grouped according to BCS on d –28 as follows: Thin (BCS ≤ 3.0), Optimal (BCS = 3.25–3.5), Over-conditioned (BCS ≥ 3.75), n = 14 each. Values are expressed as LSM. Letters "a", "b" and "c" indicate a difference ($P \leq 0.05$).

Characteristic	Group			SEM	P-value
	Thin	Optimal	Over-conditioned		
Milk					
up to d 42, kg/d	36.0	36.7	38.0	54.4	0.57
up to d 305, kg	10,079	10,639	10,824	463	0.50
ECM[1] up to d 42, kg/d	37.1[a]	38.6[ab]	42.7[b]	56.2	0.02
ECM up to d 305, kg	9,901	10,484	10,909	384	0.13
Fat up to d 42, %	4.25[a]	4.38[a]	4.99[b]	0.15	<0.01
Fat up to d 305, %	3.89	3.87	4.02	0.14	0.72
Protein up to d 42, %	3.32	3.36	3.33	0.06	0.82
Protein up to d 305, %	3.22	3.31	3.34	0.06	0.31
DMI, kg					
d –28 to d –15	14.2	13.8	12.2	0.72	0.13
d –14 to d –8	15.1[a]	14.8[ab]	12.7[b]	0.68	0.02
drop[2]	1.77	3.52	3.46	0.85	0.23
BCS					
on d –28	2.86[a]	3.33[b]	3.89[c]	0.06	<0.01
loss[3]	0.45[a]	0.64[a]	1.07[b]	0.08	<0.01

[1] Energy corrected milk, calculated according to Sjaunja *et al.* (2001)
[2] The subtract between the average DMI on d –14 to –8 and d –1
[3] Total BCS loss in the first 42 days in milk

Milk yield for the first 42 days in milk, 305-day milk and ECM production did not differ between the groups, but ECM production up to d 42 was greater in group OC compared to group T ($P < 0.05$; Table 2). OC cows had higher milk fat percentage in the first 42 days of lactation compared to O ($P < 0.05$) and T ($P < 0.01$) cows.

The BCS on d –28 was different between the groups ($P < 0.01$; Table 2) and the loss until 42 days in milk was the greatest in OC cows ($P < 0.01$).

4.2. Blood metabolites

OC cows had higher concentration of NEFA throughout the study period, differing from T and O cows on d –28 and d –14, and from T cows on d –7, d 7 and d 21 ($P < 0.05$; Figure 4). The BHB concentration was lowest on d –14 for OC cows and higher than in T cows on d 14 ($P < 0.05$). On d –21 the glucose concentration in T cows was lower than in OC cows ($P < 0.05$; Figure 4). From pre- to postpartum glucose and insulin concentrations followed the same pattern of change. No differences were recorded in the concentrations of insulin between the BCS groups. AST activity was highest in OC cows on d 7 ($P < 0.05$), on d 14 ($P < 0.01$), and higher than in T cows on d 21 ($P < 0.05$; Figure 4).

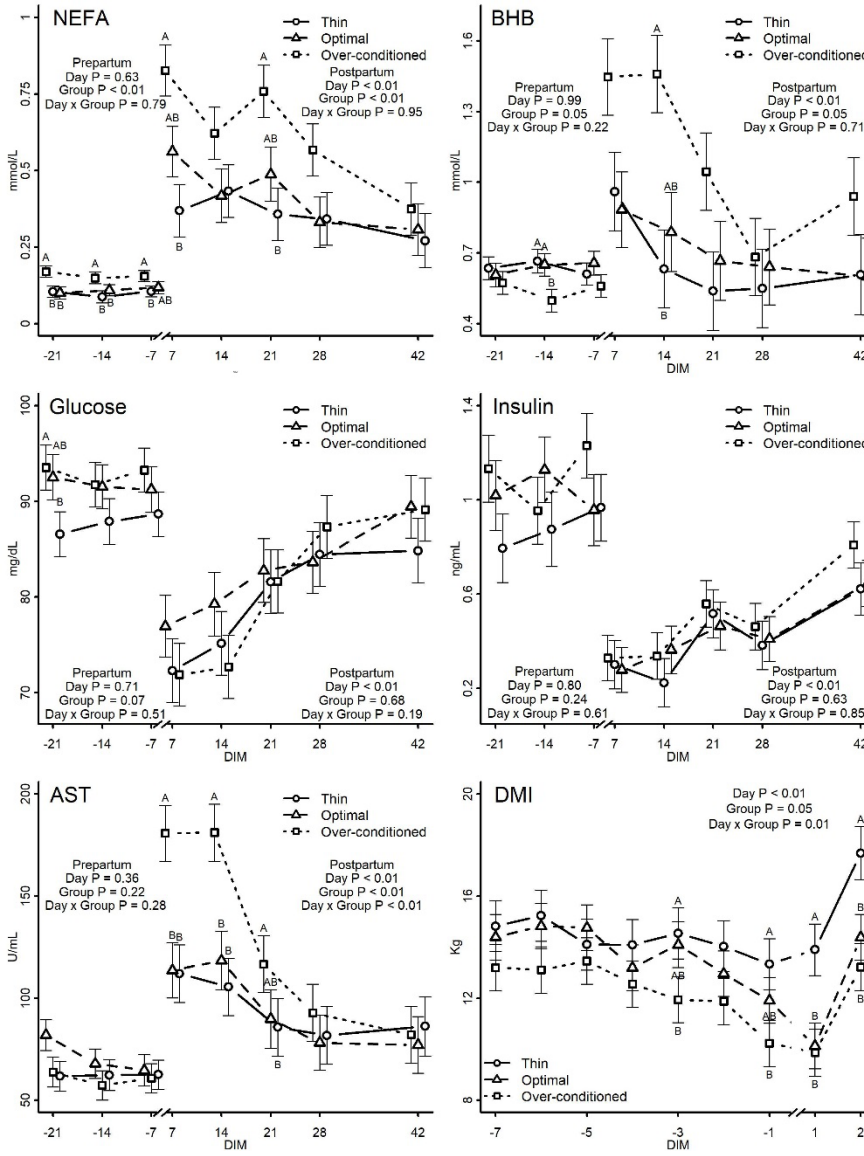


Figure 4. Concentration of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose, insulin; the activity of aspartate aminotransferase and dry matter intake (DMI) in multiparous Holstein cows grouped according to BCS on d –28 as follows: Thin (BCS ≤ 3.0), Optimal (BCS = 3.25–3.5), Over-conditioned (BCS ≥ 3.75), n = 14 each. Values are expressed as LSM ± SEM. Letters "a" and "b" indicate a difference (P ≤ 0.05).

4.3. Intravenous glucose tolerance test

In general, the infusion of glucose was associated with a rapid increase in glucose and insulin and gradual decrease in NEFA concentrations (Figure 5).

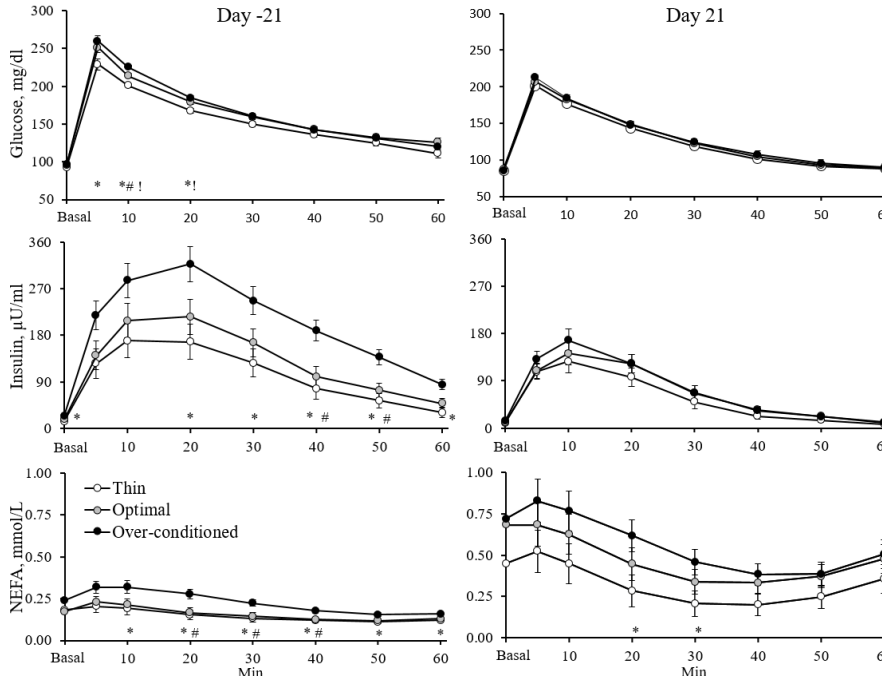


Figure 5. Concentrations of glucose, insulin and non-esterified fatty acids (NEFA) during the i.v. glucose tolerance test on d –21 and d 21 relative to calving in multiparous Holstein cows grouped according to body condition score (BCS) on d –28 as follows: Thin (BCS ≤ 3.0), Optimal (BCS = 3.25–3.5), Over-conditioned (BCS ≥ 3.75), n = 14 each. Values are expressed as LSM ± SEM. Asterisks (*) indicate a difference (P ≤ 0.05) between Thin and Over-conditioned groups, exclamation mark (!) between Thin and Optimal groups, and pound sign (#) between Optimal and Over-conditioned groups.

Basal glucose concentration did not differ between groups (Table 3), but the increment post infusion in T cows was lower compared to OC cows ($P < 0.05$). This resulted in lower glucose concentration in T cows compared to OC cows at min 5 ($P < 0.05$). All groups' glucose concentrations at min 10 differed ($P < 0.05$), T cows had the lowest and OC cows the highest values. At min 20 T cows had the lowest concentrations ($P < 0.05$). No differences in glucose concentration were recorded on d 21, but the increment was higher in OC cows compared to T cows ($P < 0.05$).

Basal insulin concentration was higher in OC compared to T cows ($P < 0.05$) on d –21. In addition, the increment was higher in OC cows compared to T cows ($P < 0.05$). Throughout the post infusion period OC cows had the highest insulin concentration, but differed from O cows at min 40 and 50 ($P < 0.05$) and from T cows at min 20 to 60 ($P < 0.05$). Insulin area under the curve (AUC) between min 5 to 60 in OC cows was higher compared to T cows ($P < 0.01$). No differences were recorded between groups on d 21.

The basal concentrations and proportional decrease of NEFA on d –21 were similar between groups, but between min 10–60 group T differed from group OC ($P < 0.05$) and between min 20–50 group O differed from OC ($P < 0.05$). Latency of NEFA was the shortest in group T compared to groups O ($P < 0.05$) and OC ($P < 0.01$) (Table 3). On d 21, the dynamics were similar between groups (Figure 4), but a difference appeared at min 20 and 30 between groups T and OC ($P < 0.05$).

4.4. Gene and protein expression

Time was a significant factor for all of the studied genes' mRNA abundance ($P < 0.05$, Figure 6). Abundance was higher on d 21 than on d –21 for *INSR*, *LIPE*, *SLC27A1*, and lower for *GLUT4*, *LEP*, *DGAT2* and *LPL*. On d –21 OC group had the lowest *DGAT2* and *LPL*, this group's mRNA abundance differed from group T ($P < 0.05$), which had the lowest expression. No differences were found between experimental groups on d 21 (Figure 6).

INSR protein expression on d –21 was higher in group T compared to group O ($P < 0.05$). Amounts of adipose tissue INSR protein did not change from d –21 to d 21 in any of the groups, but the differences between the groups disappeared on d 21 (Figure 6). GLUT4 protein expression on d –21 was smaller in group OC compared to groups T and O ($P < 0.05$). From d –21 to d 21 GLUT4 protein amount in the adipose tissue of cows from group OC did not change, but there was a clear decrease in this protein's levels in groups T and O ($P < 0.01$), but differences between the groups disappeared at d 21 (Figure 6).

Table 3. Intravenous glucose tolerance test characteristics in multiparous Holstein cows grouped according to body condition score (BCS) on d –28 as follows Thin (BCS ≤ 3.0), Optimal (BCS = 3.25–3.5), Over-conditioned (BCS ≥ 3.75), n = 14 each. Values are expressed as LSM. Letters "a", "b" indicate a difference (P ≤ 0.05).

	d –21					d 21				
	Group					Group				
Characteristic	Thin	Optimal	Over-conditioned	SEM	P-value	Thin	Optimal	Over-conditioned	SEM	P-value
Glucose										
Basal, mg/dL	92.7	95.7	96.6	2.05	0.36	84.9	87.6	85.4	3.28	0.80
Increment[1], mg/dL	139[a]	156[ab]	163[b]	6.33	0.02	116[a]	119[ab*]	127[b*]	3.03	0.01
AUC 5-60[2], mg/dL × min	3,324	3,682	3,770	150	0.09	2,114	2,161	2,348	132	0.38
Insulin										
Basal, μU/mL	13.9[a]	17.7[ab]	24.3[b]	2.32	0.00	9.63	10.7	13.5	2.26	0.72
Increment, μU/mL	166[a]	208[ab]	310[b]	33.8	0.02	119	133	154	21.5	0.74
AUC 5-60, μU/mL × min	5,308[a]	6,849[ab]	10,867[b]	1216	0.00	2,616	3,336	3,404	542	0.43
NEFA										
Basal, mmol/l	0.18	0.17	0.24	0.02	0.04	0.45	0.68	0.72	0.13	0.18
Latency[3], min	8.5[a]	16.9[b]	23.3[b]	3.00	<0.01	10.0	7.94	12.9	1.80	0.12
Decrease[4], %	38.0	34.1	33.7	4.06	0.67	46.5	55.7	43.5	5.14	0.20

[1] The difference between basal and maximal values.

[2] Area under the curve, corrected for basal values.

[3] The time from the start of glucose infusion until the concentration dropped below the basal value.

[4] The difference between basal and minimum, expressed as a percentage of the basal value.

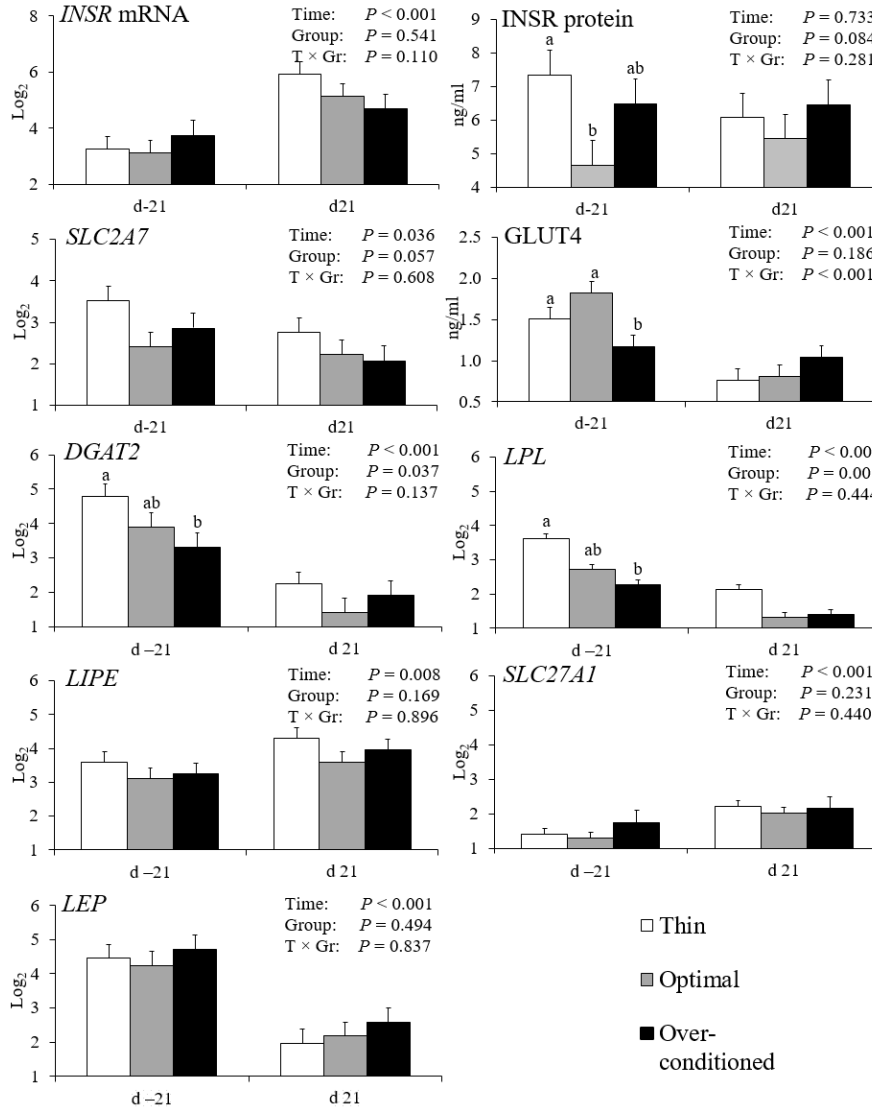


Figure 6. Adipose tissue mRNA and protein expression of insulin receptor (INSR) and glucose transporter member 4 (*SLC2A7*; GLUT4); the mRNA expression of diacylglycerol O-acyltransferase 2 (*DGAT2*), lipoprotein lipase (*LPL*), hormone sensitive lipase (*LIPE*), solute carrier family 27 member 1 (SLC27A1), leptin (*LEP*) on d –21 and d 21 relative to calving in multiparous Holstein cows grouped according to body condition score (BCS) on d –28 as follows: Thin (BCS ≤ 3.0), Optimal (BCS = 3.25–3.5), Over-conditioned (BCS ≥ 3.75), n = 14 each. Values are expressed as LSM ± SEM. Letters "a" and "b" indicate a difference (P ≤ 0.05).

4.5. Pearson correlations and regression

Positive correlations were found between insulin AUC on d –21 and plasma NEFA on d –7, d 7 (r = 0.49; 0.55, respectively, P < 0.01, Figure 7) and on d 14 (r = 0.39, *P* < 0.05, data not shown). In addition, insulin AUC on d –21 was negatively correlated with the mRNA abundance of lipogenic genes *LPL* and *DGAT2* (r = –0.32; –0.31; P < 0.05). NEFA on d 7 described 55% of BCS loss variance (P < 0.01; Figure 8).

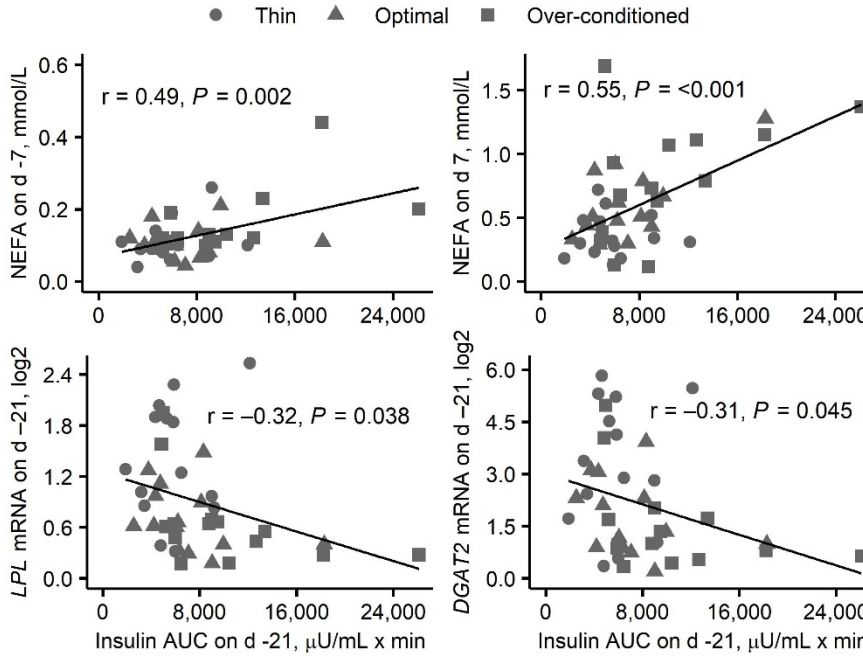


Figure 7. Pearson correlation coefficients (r) and regression lines of non-esterified fatty acids (NEFA) on d –7 and d 7, lipoprotein lipase mRNA (*LPL*) and diacylglycerol O-acyltransferase 2 mRNA (*DGAT2*) with insulin area under the curve (AUC) on d –21. Points represent the values of individual multiparous Holstein cows.

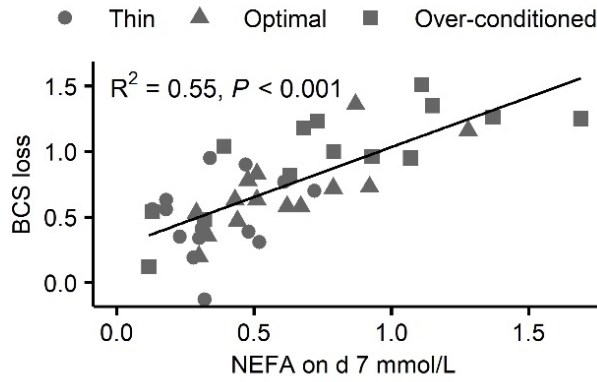


Figure 8. Pearson correlation coefficient (R^2) and regression line of body condition score (BCS) loss during the first six weeks of lactation with non-esterified fatty acids (NEFA) on d 7. Points represent the values of individual multiparous Holstein cows.

5. DISCUSSION

5.1. Effect of lactation stage on blood metabolites, IVGTT dynamics and gene expression in SAT

The magnitude of change from prepartum to postpartum in concentrations of metabolites and hormones, activity of enzymes in blood, and abundance of mRNAs and concentrations of proteins in SAT illustrate the drastic shift in cows' metabolism in order to adapt to lactation challenges.

The onset of milk secretion creates a huge demand for glucose that leads to its lower concentration in blood. In addition to low glucose, the prevalent hormonal agents (e.g. high growth hormone concentration) and negative energy balance hinder insulin secretion, causing its concentration drop in blood. Compared to d –7, glucose concentration dropped by a third and insulin concentration was halved on d 7. This status favours lipid mobilization from adipose tissue leading to high concentration of NEFA, incomplete oxidation of which is accompanied by an increase in BHB.

As expected, the infusion of glucose during IVGTT rapidly increased its concentration in blood; this in turn caused the production and release of insulin from the pancreatic β-cells. However, the response was significantly smaller postpartum compared to prepartum. This is probably due to an increase in glucose turnover rate caused by the onset of lactation. The average milk production on the day of IVGTT was 38.8 kg and for each kg of milk 72 grams of glucose is needed (Kronfeld, 1982). Assuming a steady milk production throughout the day, 116 grams of glucose was used by the udder per hour. It is thought that the udder utilizes 85% of the total glucose requirement postpartum (Bell and Baumann., 1997; Rose *et al.*, 1997); this means a total requirement of 137 g/h. In comparison, the equivalent requirement prepartum was approximately 62 g/h. Glucose infusion rate in the current experiment was 0.15 g/kg BW and the average BW of cows at the day of IVGTT prepartum and postpartum were 725 and 645 kg, respectively. Taking into account the glucose infusion rate and the average BW, 109 grams of glucose was infused prepartum and 97 grams postpartum. Thus, it was expected that the infusion would have a greater effect prepartum. The

dynamics of metabolites during IVGTT both pre- and postpartum are well in line with those reported elsewhere (Holtenius *et al.*, 2003; Mann *et al.*, 2016; Saed Samii *et al.*, 2019).

This shift in metabolism comparing prepartum and postpartum is evident at yhe cellular level in SAT as well. A decrease was observed in *SLC2A4, DGAT2* and *LPL* mRNA abundance, GLUT4 protein abundance and an increase in *LIPE* mRNA abundance on d 21 compared to d –21. These changes suggest potentially lower insulin-mediated glucose uptake (due to decreased *SLC2A4* and GLUT4), fewer lipoprotein-derived fatty acids (due to decreased *LPL*), lower intracellular (re-)esterification of fatty acids to triacylglycerol (due to the decrease in *DGAT2*), and higher hydrolysis of stored triglycerides (due to the increase in *LIPE*) in adipose tissue in early lactation compared to prepartum. In accordance, Ji *et al.* (2012) also found an overall decrease in the expression of lipogenic genes postpartum except for genes related to fatty acid uptake. This shift from lipogenesis to lipolysis results in an increase in plasma NEFA and puts a high burden on the liver to either oxidize NEFA or incorporate them into lipoproteins. Higher AST activity, a marker of liver damage, and BHB concentration in blood are clear evidence for that. The higher expression of *SLC27A1* postpartum is opposite to the changes found for the other lipogenic pathway genes (*LPL* and *DGAT2*). Alharthi *et al.* (2018) and Sadri *et al.* (2021) recently also reported the postpartum increase in *SLC27A1* expression. This suggests that not all lipogenic genes are downregulated postpartum and that there are different control mechanisms for the genes studied.

Taken together, gene and protein expression in SAT at the beginning of lactation favour lipolysis and the potential to bring energy into the cell (*SLC2A4*, *LPL,* GLUT4) and accumulate it (*DGAT2*) is lower. This in combination with low glucose and insulin concentrations, cause high NEFA and BHB concentrations and AST activity in blood.

5.2. Effect of body condition on blood metabolites, IVGTT dynamics and gene expression in SAT

5.2.1. Prepartum

The volume of adipose tissue had little effect on blood metabolites prepartum. Only NEFA was constantly higher in OC cows.

There were some discrepancies between IVGTT and weekly blood sample results on d –21 for glucose, insulin and NEFA concentrations. Glucose and NEFA concentrations between groups differed on d –21 in weekly blood samples, but did not differ according to IVGTT data (basal values). Vice versa was found for insulin. There could be several reasons for this. IVGTT was aimed to be carried out on d –21, of course this criterion was not precise for all of the animals, as calving date cannot be predicted exactly. Weekly blood samples were taken more often and are, on average, closer to the actual d –21. The IVGTT procedure is invasive and there might have been a stress response affecting the basal values. Nevertheless, the values are in line with each other and indicate higher glucose and insulin concentrations in OC cows compared to T cows and the highest NEFA concentrations in OC cows.

The NEFA concentrations were higher in OC cows throughout prepartum, but its physiological effect is probably minor, because the concentrations in all groups were well below the threshold value (0.3 mmol/L) for the prepartum period that puts the cows at risk postpartum (Ospina *et al.*, 2010). Adipose tissue volume increases mainly through the increase in the size of adipocytes (Locher *et al.*, 2015) and larger adipocytes have higher basal lipolytic rates (De Koster *et al.*, 2016). This may explain the higher NEFA concentrations in OC cows. On the other hand, the differences described may have stemmed from an increase in insulin resistance in OC cows. In support of this, Bogaert *et al.* (2018) associated high BCS prepartum with an increase in endocrine pancreas's volume and insulin secretory capacity and Schuh *et al.* (2019) found increases in glucose and insulin concentrations in over-conditioned cows.

Insulin and leptin concentrations in blood are strongly correlated (Block *et al.*, 2003; Kokkonen *et al.*, 2005; Locher *et al.*, 2015) and cows with a higher BCS have higher leptin production (Chilliard *et al.*, 2005; Schuh *et al.*, 2019). Thus, it is justified to assume higher leptin in OC cows. This would explain the lower DMI in OC cows prepartum, also seen by other authors (Daros *et al.*, 2020). However, the leptin mRNA data contradicts this assumption, as no differences were recorded between the groups. In agreement with our results, Schoenberg *et al.* (2011) did not find BCS to be an important factor for *LEP* expression. *LEP* expression in SAT is less than in other adipose depots (Depreester *et al.*, 2018) and SAT is a relatively small adipose depot (Ruda *et al.*, 2019), therefore

the contribution of the expression of *LEP* in SAT to plasma leptin concentration may also be smaller.

It has become common knowledge that a dairy cow's DMI gradually decreases up to 30% over the last three weeks of gestation (Ingvartsen and Andersen, 2000). However, the current findings do not support this notion as DMI dropped only in the final days of gestation, and this seemed to be dependent on BCS. In maize-silage based feeding systems (Janovick and Drackley, 2010; Richards *et al.*, 2020), the reported DMI drop prepartum is higher and the duration of it is longer compared to those reported in grass-based feeding systems (Agneäs *et al.*, 2003; Salin *et al.*, 2018; Trevisi *et al.*, 2015). High starch intake seems to be one of the possible reasons for DMI depression prepartum (Grummer *et al.*, 2004; Roche *et al.*, 2013). The reason could be the fact that glucose, availability of which is increased, is more tightly regulated and has more of an effect on the endocrine system (Bradford and Allen, 2007) and promotes leptin synthesis through increased insulin secretion. This is also consistent with the hepatic oxidation theory for DMI as acetate, the most dominant volatile FA from grass-based diets, is not extracted from the blood by the liver (Allen, 2020). The experimental cows of this study were fed a TMR based on grass-clover silage, so the results support the previous argument. Even though the concentrates were added to the feed for the last three weeks of gestation, the calculated starch concentration was still smaller than is usually seen in maize silage based feeding systems. Sustaining an adequate DMI of the cows during the transition period is one of the most important challenges for dairy farmers (Cardoso *et al.*, 2020). To achieve this, overconditioning and high starch intake prepartum should be avoided. The upper limit of starch concentration prepartum is, of course, beyond the scope of this thesis.

The IVGTT data confirmed the difference in insulin resistance status between the BCS groups. OC cows had slightly higher glucose increment and concentrations during the first 30 min of the IVGTT compared to T cows. This brought along a significantly higher insulin concentrations during IVGTT as evidenced by the highest insulin AUC in OC cows, which means that they had to release more insulin in order to remove the same amount of glucose from blood. High insulin AUC is an indicator of increased pancreatic insulin secretion to compensate for the peripheral IR (Bogaert *et al.*, 2018) and it is used as an IR marker (Zachut *et al.*, 2013). In general, an increase in body fatness causes an increase

in insulin resistance. There are no reported thresholds above which the insulin response can be described as pathological. In this thesis, the IR status was assessed by comparing the BCS groups. The mRNA abundance of GLUT4 (*SLC2A4*) did not differ between groups, but this was not reflected in the protein concentration, which was lowest in OC cows. GLUT4 is an insulin-dependent glucose transporter, and its lower concentration in OC cows suggests a possibly slower uptake of glucose by SAT, which may explain the higher increment of glucose, and insulin and insulin AUC in OC cows. Similarly, Liang *et al.* (2020) showed reduced GLUT4 protein in SAT of over-conditioned cows prepartum. Therefore, low GLUT4 seems to be the potential reason of IR.

Although lipolysis was low on d –21, as indicated by low NEFA concentration, insulin further repressed lipolysis as NEFA decreased during IVGTT in all groups. Even with the highest AUC, insulin did not enhance NEFA clearance in OC cows; on the contrary, the OC cows had higher NEFA values throughout IVGTT. Insulin's half-maximal and maximal inhibitory effect on lipid metabolism occurs at a considerably lower insulin concentration than that which affects on glucose metabolism (De Koster *et al.*, 2015; Petterson *et al.*, 1994). For example, De Koster *et al.* (2015) estimated that a half-maximal effect on glucose uptake occurs when insulin concentration is 76.4 μU/mL, whereas the half-maximal effect on lipolysis occurs when insulin concentration is 19.3 μU/mL. During IVGTT the insulin concentration was sufficiently high in all of the groups to elicit a maximal (or close to maximal) effect. This explains why higher insulin concentration in OC cows did not have an additional effect on NEFA and why NEFA decrease was similar in the groups. However, the latency of insulin effect on NEFA was associated with BCS group and was shorter in group T. As latency has been used to assess insulin sensitivity (Salin *et al.,* 2018), IR might be responsible for the differences observed during IVGTT.

Regarding the gene expression in SAT, the expression of lipogenic genes *LPL* and *DGAT2* were lower for OC cows on d –21, suggesting that high adipose tissue volume causes a decrease in lipogenesis in SAT, as lipogenesis is mainly controlled at the transcription level (Khan *et al.*, 2013). This is supported by similar findings by Sadri *et al.* (2021) who reported increased *LPL* expression in over-conditioned cows and De Koster *et al.* (2018) who reported a negative correlation between *DGAT2* and adipocyte size prepartum. In addition, the *LPL* and *DGAT2*

expression results may partly explain the differences in NEFA dynamics after glucose infusion and concentrations from d –21 to d –7. OC cows had a longer latency of NEFA during IVGTT, and highest NEFA from d –21 to d –7, which may have been a result of a slower elimination and re-esterification of plasma NEFA in SAT during IVGTT, thus reducing lipogenesis.

To conclude, over-conditioned cows had higher prepartum NEFA values, highest glucose peak and insulin secretion, higher NEFA concentrations and longer latency after glucose infusion, and lower abundance of *LPL* and *DGAT2* and lower GLUT4 concentration compared to thinner cows. This all indicates that high adipose tissue volume in the prepartum period lowers the insulin response in insulin-sensitive tissues.

5.2.2. Postpartum

The loss of BC during the first six weeks of lactation was related to the BCS before calving. Cows with higher BCS lost more BC postpartum, which is a well known phenomenon (Rosche *et al.*, 2009). DMI intake between OC and O cows did not differ on d 1 and d 2 postpartum, OC cows mobilized more body lipids, especially in the first weeks of lactation, that is OC cows experienced higher lipolysis regardless of DMI. In agreement, Szura *et al.* (2020) quantified the mass of subcutaneous and abdominal adipose tissue during early lactation and reported that the loss of mass is only marginally related to energy balance. Taking into account the average body weight and BCS, the estimated energy mobilized in the groups were the following: T cows – 1,080 MJ, O – 1,464 MJ, and OC – 3,096 MJ (NRC, 2001). Assuming an energy requirement of 5.25 MJ per kg of ECM (Oll and Tölp, 1995), tissue mobilization was sufficient to support 206 kg, 279 kg and 590 kg of ECM in groups T, O and OC, respectively. Indeed, total ECM production in the first six weeks was higher in OC cows. Higher production was driven mainly by a high fat content in the milk. In the first week of lactation the uptake of fatty acids from blood by the mammary gland accounts for approximately 40% of total milk fatty acids (Bell, 1995). The OC cows' high milk fat percentage is probably caused by high lipolysis and high NEFA concentrations in blood. This explanations was supported by the highest milk C18:1 *cis*-9 fatty acid concentration found in the OC group ($P < 0.01$, unpublished data).

Although OC cows' ECM production was the highest at the beginning of lactation, this was not favourable from the metabolism point of view. OC cows were clearly the most challenged as their average NEFA concentration in the first three weeks was above the risk threshold (1 mmol/L; Ospina *et al.*, 2010), BHB concentrations in the first two weeks were above the threshold for subclinical ketosis, and the activity of AST in the first two weeks was nearly twice as high as in the other groups. Therefore, the metabolism of OC cows was more unbalanced compared to the other BC groups. Due to the small number of animals in this study, the effect of BCS on health, fertility or longevity was not assessed, but the causal relationship of unbalanced metabolism at the beginning of lactation with production disease, supressed immunity, inflammation, lower fertility etc. has been explored previously (Ingvartsen and Moyes, 2013; Gross and Bruckmaier, 2019). For example, high NEFA, BHB and AST are all predictive of fatty liver disease (Bobe *et al.*, 2004).

No differences between the BC groups were observed regarding the dynamics of glucose, insulin or NEFA during IVGTT. Likewise, the mRNA abundance of *INSR, SLC2A4, DGAT2, LPL, LIPE, SLC27A1,* and *LEP* and protein concentration of INSR and GLUT4 did not differ between groups on d 21. Similarly, no difference between cows with low or high body condition was found for the abundance of: *SLC27A1* and *LIPE* by Alharthi *et al.* (2018) and Sadri *et al.* (2021); *INSR* and GLUT4 by Liang *et al.* (2020); and *LPL* and *LEP* by Sadri *et al.* (2021). The only exception was the slightly higher insulin increment in OC cows compared to T cows. Therefore, neither the differences in gene expression nor insulin signalling explain the higher lipid mobilization in over-conditioned cows.

Due to the high glucose requirement of the udder, the proportion of insulin-dependent glucose consumption by peripheral tissues in lactating cows is considered small and so differences between the groups, if existent, might be overshadowed by the insulin-independent glucose removal and were not detected. Similarly, Saed Samii *et al.* (2019) reported no differences in glucose tolerance between lean and overweight cows postpartum. And Weber at al. (2016) found no differences in insulin dependent glucose utilization and pancreatic insulin release between cows with high or low liver fat content. Holtenius *et al.* (2003) reported that glucose clearance rate was less in over-conditioned cows, and the authors attributed this to an IR in obese cows. The highest glucose

increment in OC cows, observed in the present study, could support this conclusion. However, these results are not directly comparable, as Holtenius *et al.* (2003) achieved different BCS through differential feeding, which has an impact on glucose kinetics (Schoenberg *et al.*, 2012; Petterson *et al.*, 1993).

Nevertheless, IR might be the reason for the high lipid mobilization of OC cows. The rationale behind this argument is the positive correlation between insulin AUC on d –21 and NEFA on d –7, d 7 and d 14. This means that the cows with higher insulin release during IVGTT three weeks before parturition tended to use more body lipids in the weeks around calving. Thus, the data indicate that the IR status on d –21 carries over into the later weeks of prepartum and the first weeks postpartum. This is supported by Zachut *et al.* (2013) and Zachut and Moellem (2017) who reported that cows with high weight loss postpartum had higher insulin AUC prepartum.

Considering the NEFAs associations with BCS and IR status prepartum, and the fact that NEFA on d 7 was the major determinant of the total BCS loss during the first six lactation weeks, it is clear that the success of the adaption of cows' metabolism to a new physiological state is already determined during the dry period. Overconditioning and the development of IR should be avoided. With good management practices the BCS of dried off cows can be optimized, but the reason for IR prepartum needs to be further studied, including the role of a genetic component in its development.

CONCLUSIONS

1) Overconditioning causes minor differences in glucose and NEFA concentrations during the last three weeks prepartum, which may be related to IR (I, II, III).
2) Over-conditioned cows face a more pronounced IR prepartum and its effect was evident in both glucose and lipid metabolism (I, II)
3) The expression of lipogenic genes and GLUT4 concentration in SAT were lower in over-conditioned cows three weeks before calving, which could be a potential mechanism of IR (II).
4) The onset of milk secretion creates a huge demand for nutrients and energy, leading to a decrease in glucose, and insulin concentrations, but an increase in NEFA, and BHB concentrations in blood (III).
5) Over-conditioned cows increase their energy corrected milk production during the first months of lactation, but this is at the expense of adipose tissue energy. Their adaptation to the demands of lactation was the worst of the BCS groups, and they had the most unbalanced metabolism (III).
6) Metabolic state, possibly IR, that causes more prominent insulin release after glucose infusion three weeks before parturition is related to higher lipid mobilization up to two weeks postpartum and is associated with lower expression of lipogenic genes in subcutaneous adipose tissue (II).
7) At the beginning of lactation lipolysis in SAT is supported by lower expression of mRNA/protein responsible for bringing in (*SLC2A4*, *LPL,* GLUT4) and accumulating energy (*DGAT2*) (I, II).
8) Adipose tissue volume did not affect the mRNA expression of leptin in SAT (II).
9) There were no differences between the groups' postpartum gene expression and metabolites' dynamics after glucose infusion. This means that the difference in IR state between groups had disappeared by the third lactation week (I, II).
10) Concentrations of NEFA in the first week of lactation, dependent on IR status in the dry period, describe most of the variation in BCS loss during the first six weeks postpartum. Thus, metabolic processes during the dry period and in the first week of lactation are important determinants for the success of lactation (III).

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SUMMARY IN ESTONIAN

Toitumuse, lipomobilisatsiooni ja insuliiniresistentsuse seosed

Piimatootmine muutus 20. sajandi esimestel aastakümnetel Eestile väga oluliseks majandusharuks, edule aitas kaasa tulus või eksport. Näiteks 1931. aastal moodustas või eksport 75% kogu põllumajandustoodete ja 36% kogu Eesti ekspordi väärtusest. Eksportturgude leidmine on siiani Eesti piimandussektorile oluline, sest piima isevarustatuse tase on püsinud 160% lähedal. Aastal 2020 oli Eestis jõudluskontrollis olevate lehmade keskmine aastatoodang 10 400 kg, vaid 20 aastat tagasi oli see poole väiksem. Toodangu kasvu pidurdumist lähiaastatel näha ei ole, sest 2017. aastal teatati USAs uuest maailma rekordlehmast, kes lüpsis 365 päeva jooksul üle 35 000 kg piima. Ka Eestis pole laktatsioonitoodangud üle 20 000 kg haruldased. Suur toodang paneb täiendava koormuse lehmade ainevahetusele, mille protsesside parem mõistmine on tähtis, et tagada lehmade hea tervis, heaolu ja pikk eluiga. Üleminekuperioodil (ingl *transition period),* kolm nädalat enne kuni kolm nädalat pärast poegimist, on piimalehma ainevahetus iseäranis koormatud. Piimasünteesist põhjustatud toitainete tarbe mitmekordistumine ning kehavarude arvelt piima tootmine, millele lisandub poegimisest tingitud põletikuseisund ja langenud immuunsus, muudavad lehma vastuvõtlikuks erinevatele terviseprobleemidele. Ligi 75% haigusjuhtudest karjas esineb esimesel laktatsioonikuul ning ligi 80% praakimistest on tingitud haigustest.

Ainevahetusprotsesside regulatsioonis on suur tähtsus pankreases sünteesitaval signaalhormoonil insuliinil. Insuliin aktiveerib organismis energia salvestamisega seotud protsesse. Nii aktiveerib insuliin glükoosi viimise verest rakku, stimuleerib lipogeneesi – varurasvade sünteesi – ja inhibeerib lipolüüsi – varurasvade kasutamist. Insuliini signaal algab tema kinnitumisega raku membraanil olevale retseptorile (INSR), seejärel vallandub rakusisene signaali ülekanne, mille tulemusena suureneb glükoosi transportvalku kodeeriva geeni *(SLC2A4)* ja vastava valgu ekspressioon. Transportvalgud (glükoosi transporter 4; GLUT4) ühinevad raku membraaniga ja toovad glükoosi läbi membraani rakku. Insuliiniresistentsus (IR) on seisund, mille puhul insuliini signaali ülekanne organismis on pärsitud. IRi määramise kõige praktilisem viis piimalehmadel on intravenoosne glükoosi tolerantsi test (IVGTT), mille käigus viiakse verre kindel kogus glükoosi ja järgnevalt hinnatakse

organismi vastust – vere glükoosi, insuliini jt metaboliitide glükoosi infusiooni järgset dünaamikat.

Laktatsiooni alguses soosib lehmade hormonaalne tasakaal lipolüüsi ja insuliin on selle protsessi põhiline antagonist. Kui insuliini signaali ülekanne rasvkoes on häiritud, siis on tagajärjeks sellesse varutud energia intensiivne kasutamine. Lipolüüsi (hormoontundlik lipaas – *LIPE*) ja lipogeneesi (lipoproteiini lipaas – *LPL,* rasvhappeid transportiv valk 1 – SLC27A1, diglütseriidi atsüültransferaas – DGAT2) reguleerivate geenide ekspressiooni vahekord rasvarakus peegeldab nende protsesside tasakaalu. Rasvkoe energia kasutamise markeriks veres on esterifitseerimata rasvhapped (NEFA) ja β-hüdroksübutüraat (BHB), nende kõrge kontsentratsioon veres on suur riskitegur ainevahetushaiguste väljakujunemiseks. Rasvkoe partnerorgan energiaainevahetuse tasakaalu hoidmisel on maks, mille ülekoormust saab hinnata muuhulgas aspartaadi aminotransferaasi (AST) aktiivsuse alusel. Lisaks energia talletamisele on rasvkoel keskne roll nii ainevahetuses kui immuunsüsteemis. Rasvkoes sünteesitakse näiteks väikeseid peptiide, mis annavad edasi signaale organismi teistele kudedele ja rakkudele. Üks sellistest peptiididest, leptiin (LEP), vähendab söömust ja inhibeerib insuliini sünteesi. Seetõttu on rasvkoes toimuvate protsesside uurimine piimalehmadel suure tähtsusega.

Eesti Maaülikooli söötmisteaduse õppetooli ainevahetuse töörühma teadlased keskendusid aastatuhande vahetusel oma teadustöös piimalehmade üleminekuperioodi ja sellega kaasneva energiabilansi muutusega seotud probleemide uurimisele. Kasutusele võeti toitumushinde (TH) (ingl *body condition score* – *BCS*) määramine, mis võimaldab nahaaluse rasvkoe hulga alusel hinnata kogu keha energiatagavara. Jätkuva teadustöö käigus on leitud sobiv TH poegimisel, TH seosed sigimisega, toodanguga, vere metaboliitidega, insuliiniresistentsusega jne. Käesolev doktoriväitekiri jätkab õppetooli varasemat teadustööd, ühendades omavahel toitumushinde, insuliiniresistentsuse ja rasvkoes molekulaartasemel toimuva ning ühendades kolme artikli tulemused. Teaduskirjanduses on vähe süsteemse lähenemisega uuringuid, mis seoks TH ja IRi rasvkoe geenidekspressiooniga. Lisaks pole selget arusaamist IRi tekkepõhjustest ja toimemehhanismidest molekulaartasemel. Käesolev doktoriväitekiri panustab teadmiste edenemisse nimetatud teemadel.

Doktoriväitekirja hüpotees oli, et toitumus enne poegimist ja poegimisjärgne varurasvade kasutamine on seotud organismi insuliiniresistentsusega ja glükoosi ning lipiidide ainevahetust reguleerivate geenide ekspressiooniga rasvkoes. Sellest lähtuvalt seati eesmärgiks uurida eesti holsteini tõugu lehmade kinnisperioodi TH mõju:

- söömuse, TH, vere metaboliitide (glükoos, insuliin, NEFA, BHB, AST) ja piimatoodangu dünaamikale laktatsiooni alguses (III);
- glükoosi, insuliini ja NEFA dünaamikale IVGTT ajal kolm nädalat enne ja pärast poegimist (I, II);
- glükoosi ainevahetust reguleerivate geenide *INSR* ja *SLC2A4* ekspressioonile ja vastavate proteiinide kontsentratsioonile nahaaluses rasvkoes kolm nädalat enne ja pärast poegimist (I);
- lipiidide ainevahetust reguleerivate geenide *SLC27A1, LEP, LPL, DGAT2, LIPE* ekspressioonile nahaaluses rasvkoes kolm nädalat enne ja pärast poegimist (II).

Doktoriväitekirja eksperimentaalne osa viidi läbi Eesti Maaülikooli Märja katselaudas 42 eesti holsteini piimalehmaga, kes jagati poegimiseelse TH alusel kolme võrdse suurusega rühma: TH ≤ 3,0 (kõhn); TH = 3,25–3,5 (paras); TH ≥ 3,75 (paks). Kolm nädalat enne ja pärast poegimist tehti katselehmadel insuliiniresistentsuse hindamiseks IVGTT. Lehmade verre viidi 40% lahusena 0,15 grammi glükoosi kilogrammi kehamassi kohta. Arvestades infusiooni algust nullpunktiks, võeti vereproov minutitel -5, 5, 10, 20, 30, 40, 50, 60 ja neist määrati glükoosi, NEFA (spektrofotomeetriliselt) ning insuliini (radioimmuunanalüüs (RIA)) kontsentratioon. IVGTTga samal päeval, kolm nädalat enne ja pärast poegimist, võeti päraluunukilt rasvkoe proov, millest määrati glükoosi ainevahetust reguleerivate geenide *SLC2A4* ja *INSR* ekspressioon (pöördtranskriptaas polümeraasi ahelreaktsiooni meetodiga (RT-qPCR)) ja vastavate valkude kontsentratsioon (ensüüm-seotud immuunsorbent määramine (ELISA)) ning lipiidide ainevahetust reguleerivate geenide *SLC27A1, LEP, LPL, DGAT2, LIPE* ekspressioon. Nädalatel -3, -2, -1, 1, 2, 3, 4, 6 sõltuvalt poegimisest võeti katseloomadelt ainevahetusliku seisundi hindamiseks vereproov, millest määrati spektrofotomeetriliselt NEFA, BHB, glükoosi kontsentratsioon ja ASTi aktiivsus ning ELISA meetodil insuliini kontsentratsioon. Lisaks määrati lehmade söömus enne poegimist ja esimesel kahel laktatsioonipäeval ning piimatoodang esimese kuue laktatsiooninädala ja terve laktatsiooni lõikes. Andmete

statistiliseks analüüsiks kasutati programmi „R“. Hindamaks TH rühma mõju uuritavatele muutujatele koostati lineaarsed segamudelid, kasutades paketi „lm4“ funktsiooni „lme“.

Töö tähtsamad tulemused ja järeldused

1. Glükoosi infusioon enne poegimist põhjustas paksudel lehmadel intensiivsema insuliini sünteesi ja sekretsiooni pankreasest. Seega oli neil loomadel vaja glükoosi rakku viimiseks rohkem insuliini kui väiksema rasvkoe hulgaga loomadel. Samuti algas neil glükoosi infusiooni järgne NEFA langus, mis näitab insuliinist põhjustatud lipolüüsi vähenemist, hiljem. Sellest järeldub, et paksud lehmad olid insuliini toimele resistentsemad. Samal ajal oli paksude lehmade nahaaluses rasvkoes väiksem GLUT4 kontsentratsioon ja madalam lipogeensete geenide (*LPL* ja *DGAT2*) ekspressioon.

2. Poegimisjärgne piimasünteesi algus tõi kaasa märkimisväärse toitainete ja energia tarbe kasvu, mistõttu vähenes glükoosi ja insuliini, kuid suurenes varurasvade kasutamise intensiivsust iseloomustavate markerite NEFA ja BHB kontsentratsioon veres.

3. Paksude lehmade esimese kuue laktatsiooninädala energia järgi korrigeeritud piimatoodang oli keskmiselt 42,7 kg, mis on 3,9 kg võrra suurem parajate ja 5,6 kg suurem kõhnade lehmade toodangust. Suurema toodangu saavutasid paksud lehmad rasvkoes talletatud energia suurema kasutamise arvel. Seejuures oli nende loomade kohanemine laktatsiooni alguse intensiivse ainevahetusliku koormusega kõige halvem – esimestel laktatsiooninädalatel oli nende keskmine NEFA kontsentratsioon veres üle piirväärtuse (1 mmol/L), mis tõstab haiguste esinemise riski, BHB kontsentratsioon üle subkliinilise ketoosi piirväärtuse (1,2 mmol/L) ja maksakahjustuse indikaatori ASTi aktiivsus üle kahe korra suurem võrreldes teiste rühmadega.

4. Insuliiniresistentsuse ulatus kolm nädalat enne poegimist oli seotud intensiivsema varurasvade kasutamisega esimestel laktatsiooninädalatel, mis omakorda seostub lipogeensete geenide madalama ekspressiooniga nahaaluses rasvkoes.

5. NEFA kontsentratsioon laktatsiooni alguses sõltub IRist ja toitumusest kinnisperioodil. Seega määrab juba kinnisperioodi ainevahetuslik seisund suuresti laktatsiooni alguse ainevahetusliku seisundi. NEFA kontsentratsioon esimesel laktatsiooninädalal kirjeldab suurema osa toitumushinde esimese kuue nädala languse variatsioonist. See näitab, et juba laktatsiooni esimesel nädalal toimuv määrab omakorda ära, kui palju lehm järgnevatel nädalatel rasvkoe energiat kasutab.

Praktilised soovitused

Töö tulemused näitavad selgelt, et loomade liigne toitumus poegimisel on lehmade ainevahetusele koormav ja suurendab haiguste esinemuse riski. Süsteemne toitumuse hindamine võimaldab hinnata söötmise taset ja annab võimaluse vältida lehmade liigset toitumust. Arvestades, et katsesse võeti loomad, kelle toitumus kinnisperioodil ei muutunud, tuleb juba laktatsiooni lõpus lehmi sööta viisil, mis tagaks sobiva toitumuse poegimiseks.

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I

ORIGINAL PUBLICATIONS

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Adipose tissue insulin receptor and glucose transporter 4 expression, and blood glucose and insulin responses during glucose tolerance tests in transition Holstein cows with different body condition

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ABSTRACT

Glucose uptake in tissues is mediated by insulin receptor (INSR) and glucose transporter 4 (GLUT4). The aim of this study was to examine the effect of body condition during the dry period on adipose tissue mRNA and protein expression of INSR and GLUT4, and on the dynamics of glucose and insulin following the i.v. glucose tolerance test in Holstein cows 21 d before (d −21) and after (d 21) calving. Cows were grouped as body condition score (BCS) ≤3.0 (thin, T; n = 14), BCS = 3.25 to 3.5 (optimal, O; n = 14), and BCS ≥3.75 (overconditioned, OC; n = 14). Blood was analyzed for glucose, insulin, fatty acids, and β-hydroxybutyrate concentrations. Adipose tissue was analyzed for INSR and GLUT4 mRNA and protein concentrations. During the glucose tolerance test 0.15 g/kg of body weight glucose was infused; blood was collected at −5, 5, 10, 20, 30, 40, 50, and 60 min, and analyzed for glucose and insulin. On d −21 the area under the curve (AUC) of glucose was smallest in group T (1,512 ± 33.9 mg/dL × min) and largest in group OC (1,783 ± 33.9 mg/dL × min), and different between all groups. Basal insulin on d −21 was lowest in group T (13.9 ± 2.32 μU/mL), which was different from group OC (24.9 ± 2.32 μU/mL. On d −21 the smallest AUC 5–60 of insulin in group T (5,308 ± 1,214 μU/mL × min) differed from the largest AUC in group OC (10,867 ± 1,215 μU/mL × min). Time to reach basal concentration of insulin in group OC (113 ± 14.1 min) was longer compared with group T (45 ± 14.1). The *INSR* mRNA abundance on d 21 was higher compared with d −21 in groups T (d −21: 3.3 ± 0.44; d 21: 5.9 ± 0.44) and O (d −21: 3.7 ± 0.45; d 21: 4.7 ± 0.45). The extent of INSR protein expression on d −21 was highest in group T (7.3 ± 0.74 ng/mL), differing from group O (4.6 ± 0.73 ng/mL), which had the lowest expression. The amount of GLUT4 protein on d −21 was lowest in group OC (1.2 ± 0.14 ng/mL), different from group O (1.8 ± 0.14 ng/mL), which had the highest amount, and from group T (1.5 ± 0.14 ng/mL). From d −21 to 21, a decrease occurred in the GLUT4 protein levels in both groups T (d −21: 1.5 ± 0.14 ng/mL; d 21: 0.8 ± 0.14 ng/mL) and O (d −21: 1.8 ± 0.14 ng/mL; d 21: 0.8 ± 0.14 ng/mL). These results demonstrate that in obese cows adipose tissue insulin resistance develops prepartum and is related to reduced GLUT4 protein synthesis. Regarding glucose metabolism, body condition did not affect adipose tissue insulin resistance postpartum.
Key words: insulin receptor, glucose transporter 4, glucose tolerance test, adipose tissue, body condition score

INTRODUCTION

Transition from pregnancy to lactation is associated with important readjustments in metabolism of dairy cows. Due to the onset of milk synthesis, requirements for energy and nutrients, especially for glucose, increase markedly after calving, leading to negative energy balance (**NEB**; Bell, 1995). To compensate for the energy and nutrient deficiency, large quantities of fatty acids are mobilized from adipose tissue (**AT**), which, with concurrent low glucose availability, will support the production of BHB and may lead to the development of ketosis (Oetzel, 2007). Insulin and sensitivity of tissues to insulin play a central role in the adjustment of energy partitioning between tissues and in balancing lipogenesis and lipolysis (Bell and Bauman, 1997). In

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dairy cows, insulin resistance (**IR**) develops during late pregnancy and represents an important homeorhetic adaptation, canalizing metabolism to use energy, mainly stored in AT, to support body functions and to minimize glucose consumption in peripheral tissues, sparing it for milk synthesis under conditions of nutrient and energy deficiency (Bell, 1995; Salin et al., 2012; De Koster and Opsomer, 2013; Zachut et al., 2013).

In general, insulin activates pathways responsible for energy storage within the body [e.g., glucose uptake, lipogenesis, and glycogenesis (Bell and Bauman, 1997)]. In AT, insulin signal transduction starts with binding insulin to its receptor (**INSR**). The consequent intracellular cascade, mainly through the PI3K/AKT/mTOR pathway, promotes the expression, translocation, and fusion with cell membrane of insulin-dependent glucose transporters 4, responsible for insulin-induced glucose uptake from blood in adipose and muscle tissue. Therefore, INSR and GLUT4, incorporated in the membrane, represent the start and end of insulin signaling responsible for facilitated cellular glucose uptake (Lewis et al., 2002).

The plane of nutrition as a factor influencing insulin sensitivity (Holtenius et al., 2003; Schoenberg et al., 2012) and insulin signaling (Mann et al., 2016b) in cows has been extensively investigated. Regarding body fat reserves, both suboptimal body condition and overcondition before parturition are associated with poor adaptation in the subsequent lactation, leading to an increased incidence of metabolic disorders such as ketosis and fatty liver (Drackley, 1999; Bobe et al., 2004; Goff, 2006; Roche et al., 2013a,b), impaired fertility (Samarütel et al., 2008a,b; Roche et al., 2009), and more pronounced IR (Holtenius and Holtenius, 2007; Jaakson et al., 2013). According to De Koster and coworkers (De Koster et al., 2015, 2016a), development of IR in overconditioned cows at the end of the dry period is associated rather with glucose than lipid metabolism. However, despite intense research work, and due to differences in experimental conditions, design, and aims, a clear understanding regarding cause-and-effect relationships between the IR and the physiological and metabolic status of cows, as well as understanding about the molecular mechanisms of insulin signaling are still lacking. Moreover, the number of studies characterizing protein expression of INSR and GLUT4 in AT is limited (Mann et al., 2016b). No integrated studies are available describing AT INSR and GLUT4 expression in relation to BCS both at the mRNA and protein levels.

In this study we hypothesized that the development and extent of IR during the transition period, mediated by the expression and function of INSR and GLUT4, are related to the amounts of body fat reserves in the dry period. Therefore, the aim of this study was to examine the effect of BCS during the dry period on the mRNA and protein expressions of INSR and GLUT4 in s.c. AT, in addition to the dynamics of insulin and glucose following intravenous glucose infusion in Holstein dairy cows 21 d before (d −21) and after (d 21) calving.

MATERIALS AND METHODS

Experimental Design and Animals

The study was carried out on 42 multiparous Holstein cows on the experimental farm of the Estonian University of Life Sciences, which has a herd size of about 120 cows and with average annual milk production of about 9,200 kg per cow. Cows were indoor housed in freestall barns with rubber mats and sawdust bedding and fed TMR ad libitum. Lactating cows were milked twice a day. Three experimental groups were established and proportionally assigned according to the blocked design with each block consisting of 3 cows over 2 consecutive years on the basis of cows' BCS (Edmonson et al., 1989) 28 d before expected calving (d −28) as follows: BCS ≤3.0 (2.25–3.00; thin, **T**; n = 14), BCS = 3.25 to 3.5 (optimal, **O**; n = 14), and BCS ≥3.75 (3.75–4.50; overconditioned, **OC**; n = 14). Parity distribution was different between groups T [2nd to 5th (2.6) parity] and OC [2nd to 6th (3.7) parity; $P = 0.006$]; group O [2nd to 6th (3.2) parity] did not differ from other groups. Fortnightly assessment of the potential experimental cows' BCS began at drying off, on an average 54 (52–59) d before expected calving. Cows with an appropriate BCS, and who had maintained their BCS from drying off until d −28, were assigned to the study blocks. Such cows were removed from the dry cow barn to the tiestall housing to adjust to the experimental conditions. After calving, from the seventh milking, cows were removed to a freestall barn with attached milking parlor.

The European Council Directive regarding the protection of animals, and the Estonian Animal Protection Act, have been complied with in this experiment. The study has been approved by the Committee for Conducting Animal Experiments at the Estonian Ministry of Agriculture.

Feeds and Feeding

Cows were fed grass silage, hay, corn meal, barley meal, heat-treated rapeseed cake, and mineral feeds as TMR twice daily ad libitum around 0530 and 1430 h. Depending on physiological stage and requirements, cows were fed 5 rations differing in chemical composition and nutritive value (Table 1). Rations were calcu-

lated according to Estonian feeding recommendations: ME according to Oll (1995), MP on the basis of equations used in Finland (Tuori et al., 1996), modified to suit Estonian conditions as described by Kärt et al. (2002). At drying off, cows were moved into a dry cow barn and offered a far-off dry cow diet containing 8.7 MJ of ME and 73 g of MP/kg of DM. The same diet was continued to be fed after moving the cows into the tiestall barn and entering the study, from d −28 until d −15. From d −14 until calving, a close-up dry cow diet containing 10.1 MJ of ME and 87 g of MP/kg of DM was fed. After calving, until d 6 of lactation, lactation diet 1 was provided, which differed from the close-up diet only in its mineral composition. From d 7 until d 14 lactation diet 2, with an ME content of 10.9 MJ and MP content of 98 g per kg of DM was offered. Lactation diet 3, fed from d 15 onward, containing 11.3 MJ of ME and 104 g of MP per kg of DM.

Feed Sampling and Analyses

The feeding data given in Table 1 represent means for the whole 2-yr study period. Silage samples were taken twice weekly. Other feeds were sampled on a batch basis. All samples were analyzed for DM, whereas chemical composition was analyzed using methods approved by the AOAC (2005) and nutritive value (ME, MP) was calculated once weekly for silage samples, or on the batch basis for other feeds. If necessary, the proportions of the rations' ingredients were corrected to meet required composition and nutritive values.

Milk Sampling and Analyses, Calculation of ECM Yield, and Energy Balance

The cows were milked twice daily at 0500 and 1500 h. Milk yields were recorded electronically at each milking, using DeLaval's Alpro software for Windows in a DeLaval milking parlor (DeLaval, Tumba, Sweden). Morning and evening milk samples were collected on Sundays and Thursdays from March 2013 to April 2014 using in-line milk meters (MM27BC, DeLaval) and in addition to these days, samples were also collected on Tuesdays from May 2014 to December 2015. Samples from 2 consecutive milkings were pooled per cow. One milk aliquot (40 mL) of each pooled milk sample was stabilized with bronopol (Broad Spectrum Microtabs, D&F Control Systems Inc., Norwood, MA) and was analyzed for fat, protein, and lactose contents with an automatic infrared milk analyzer (System FT+, Foss Electric, Hillerød, Denmark) in the Milk Analysis Laboratory of Estonian Livestock Performance Recording Ltd. The ECM yields were calculated according to the method described by Saunja et al. (1991). The following characteristics were calculated for ECM, milk fat, protein, and lactose: yields (kg) and contents (%) for fat, protein, and lactose on d 21; yields (kg/d) and contents (%) for fat, protein, and lactose up to d 21; and yields (kg) up to d 21. Starting from the seventh milking, after removal to a freestall barn with attached milking parlor, the cows were weighed twice per day. The average BW of the BSC groups on d 5, after removal to a freestall barn, was as follows: T, 610 ± 48.0; O, 669 ± 87.2; and OC, 728 ± 92.2 kg. The energy balance of the cows was estimated based on effective energy system (Emmans, 1994) and frequent BW measurements and BCS according to Thorup et al. (2012). To characterize cows' energy status, energy balance on d 21 (MJ/d) and from parturition to d 21 (MJ/d), total energy deficit from parturition to d 21 (MJ), and nadir of NEB (MJ) were calculated (Table 2).

Blood Sampling, Glucose Tolerance Test, and Laboratory Analyses

Blood samples for metabolic profiling were taken on d −21 ± 0.6 and d 21 ± 0.2 at around 1000 h before AT biopsy and glucose tolerance test (**GTT**), from the coccygeal vein, and collected into sterile vacuum tubes with Li-heparin (Vacuette, Greiner Bio-One International GmbH, Kremsmünster, Austria) and kept at +4°C. Plasma was separated by centrifugation (5,000 × *g*, 15 min, +4°C) within 4 h after sampling, and stored at −80°C until analysis. Insulin was analyzed by bovine-optimized sandwich ELISA (catalog no. 10–1201–01; Mercodia AB, Uppsala, Sweden), with the detection limit of 0.025 ng/mL, on microplate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland); results were calculated using cubic spline regression (Magellan data analysis software; Tecan Group Ltd.). To ensure consistency of the results low (5.1 μU/mL) and high (41.1 μU/mL), control samples for insulin measurement were prepared by pooling plasmas from 4 cows with low and from 4 cows with high insulin concentrations, respectively. Interassay coefficients of variation (**CV**) were determined by analyzing the low and high control samples in 6 ELISA plates in duplicate. For determination of intraassay CV, the control samples were run in 6 replicates in duplicate in 1 ELISA plate. The interassay CV for low and high control samples were 4.6 and 6.6%, respectively; the intraassay CV were 4.2 and 4.1%, respectively. Glucose was analyzed by enzymatic-colorimetric method (product code XSYS 012, ERBA Diagnostics Mannheim GmbH, Germany), fatty acids by enzymatic-colorimetric method (catalog no. FA 115, Randox Laboratories Ltd., Crumlin, UK) and BHB by kinetic-enzymatic UV-method (catalog no. RB 1007; Randox Laboratories Ltd.) on an auto-

Table 1. Feed ingredients and chemical composition (mean ± SD) of TMR (g/kg) fed to experimental cows

	Ration				
Item	Far-off dry cow	Close-up dry cow	Lactation diet 1 from calving till d 6	Lactation diet 2 from d 7 to 14	Lactation diet 3 from d 15
Ingredient					
Grass silage	955 ± 81	599 ± 71	604 ± 72	460 ± 49	384 ± 37
Hay	33.6 ± 81	28.3 ± 69	28.6 ± 70	20.3 ± 49	15.6 ± 37
Barley meal	—	301 ± 10	303 ± 10	309 ± 0.1	296 ± 0.2
Corn meal	—	—	—	64.3 ± 0.0	120 ± 0.1
Heat-treated rapeseed cake	—	47.1 ± 0.6	47.5 ± 0.6	129 ± 0.0	168 ± 0.1
Mineral-vitamin feed	11.7 ± 0.1[1]	7.82 ± 0.2[1]	10.5 ± 0.3[2]	8.58 ± 0.0[2]	7.99 ± 0.0[2]
Anionic mineral feed[3]	—	10.4 ± 0.2	—	—	—
Limestone	—	6.26 ± 0.2	—	4.29 ± 0.0	4.41 ± 0.7
Sodium chloride	—	—	5.78 ± 0.1	4.72 ± 0.0	4.40 ± 0.0
DM	348 ± 78	435 ± 64	433 ± 64	483 ± 53	511 ± 45
Chemical composition in DM					
OM	893 ± 9.0	904 ± 5.9	911 ± 6.0	918 ± 4.5	923 ± 3.9
CP	131 ± 10.5	144 ± 7.2	145 ± 7.3	161 ± 5.6	169 ± 4.8
MP	72.8 ± 3.3	86.6 ± 2.1	87.3 ± 2.1	97.9 ± 1.6	104 ± 1.3
Protein balance value	6.22 ± 8.2	−0.37 ± 5.6	−0.37 ± 5.6	0.97 ± 4.4	0.46 ± 3.7
ME (MJ)	8.70 ± 0.3	10.1 ± 0.2	10.2 ± 0.2	10.9 ± 0.1	11.3 ± 0.1
Crude fat	29.9 ± 2.2	30.2 ± 1.4	30.5 ± 1.4	37.8 ± 1.1	42.0 ± 0.9
Crude fiber	285 ± 24	198 ± 15	200 ± 15	171 ± 13	153 ± 11
NDF	534 ± 51	455 ± 32	458 ± 32	410 ± 25	380 ± 22
ADF	387 ± 47	278 ± 29	280 ± 29	242 ± 22	220 ± 18
Calcium	11.4 ± 1.9	10.3 ± 1.3	9.33 ± 1.3	9.54 ± 1.0	8.97 ± 0.8
Phosphorus	3.42 ± 0.4	4.06 ± 0.2	4.03 ± 0.2	4.48 ± 0.2	4.77 ± 0.2

[1]Composition (as-fed basis): 170 g/kg of Ca; 50 g/kg of P; 30 g/kg of Na; 140 g/kg of Mg; 30 g/kg of S; 1,000 mg/kg of Cu; 4,500 mg/kg of Zn; 4,000 mg/kg of Mn; 40 mg/kg of Se; 50 mg of Co; 200 mg of I; 800,000 IU/kg of vitamin A; 190,000 IU/kg of vitamin D; and 8,000 IU/kg of vitamin E.

[2]Composition (as-fed basis): 150 g/kg of Ca, 35 g/kg of P, 75 g/kg of Na, 90 g/kg of Mg, 1 g/kg of S, 4,000 mg/kg of Cu, 6,667 mg/kg of Zn, 6,452 mg/kg of Mn, 94 mg/kg of Se, 109 mg of Co, 650,000 IU/kg of vitamin A, 150,000 IU/kg of vitamin D, and 4,000 IU/kg of vitamin E.

[3]Composition (as-fed basis): 9 g/kg of Ca, 1 g/kg of P, 5 g/kg of Na, 100 g/kg of Mg, 1,000 mg/kg of Cu, 5,000 mg/kg of Zn, 2,000 mg/kg of Mn, 27 mg/kg of Se, 40 mg of Co, 100 mg of I, 1,000,000 IU/kg of vitamin A, 60,000 IU/kg of vitamin D, and 10,000 mg/kg of vitamin E, and 100,000 μg/kg of biotin.

Table 2. Least squares means with pooled SEM for BCS, energy balance (EB), and milk production characteristics in experimental groups

	Group				
Characteristic	Thin	Optimal	Overconditioned	Pooled SEM	*P*-value
BCS					
d −21	2.94[a]	3.36[b]	3.80[c]	0.07	<0.001
d 21	2.63[a]	2.94[b]	3.20[c]	0.07	<0.001
Body condition loss from d −21 to 21	0.27[a]	0.38[a]	0.66[b]	0.05	<0.001
EB					
d 21 (MJ of EE[1]/d)	−24.9[a]	−41.5[a]	−79.2[b]	9.32	<0.001
From parturition to d 21 (MJ of EE/d)	−54.4[a]	−75.6[a]	−118.9[b]	11.8	<0.001
Total deficit: d 1 to 21 (MJ of EE)	−1,006[a]	−1,431[a]	−2,282[b]	226	<0.001
Nadir of negative EB (MJ of EE/d)	−109*	−130	−196*	24.6	0.039
Milk and milk component					
ECM yield on d 21 (kg)	41.1	41.8	44.7	1.76	0.328
Milk yield on d 21 (kg)	38.6	38.4	39.3	1.74	0.929
Fat content on d 21 (%)	4.09[a]	4.25[ab]	4.75[b]	0.18	0.017
Protein content on d 21 (%)	3.22	3.29	3.26	0.07	0.646
Lactose content on d 21 (%)	4.89	4.92	4.81	0.04	0.117
ECM yield up to d 21 (kg/d)	38.3	40	42.8	1.97	0.285
Milk yield up to d 21 (kg/d)	33.0	33.3	33.7	1.64	0.958
Fat content up to d 21 (%)	4.79[a]	5.02[a]	5.75[b]	0.19	<0.001
Protein content up to d 21 (%)	3.61	3.62	3.58	1.02	0.889
Lactose content up to d 21 (%)	4.69	4.68	4.59	0.05	0.275

[a–c]LSM with different letters within a row differ ($P \leq 0.05$).

[1]EE = ether extract.

*LSM with asterisk within a row tend to differ ($P \leq 0.1$).

matic analyzer (ERBA XL300, Randox Laboratories Ltd.). For glucose, fatty acids, and BHB, intraassay CV were determined by analyzing plasma in 20 replicates in 3 duplicates; interassay CV were calculated on the basis of 3 duplicates. The interassay CV were 4.8, 8.3, and 6.1%, and intraassay CV 4.9, 6.1, and 5.7% for glucose, fatty acids, and BHB, respectively. For quality assessment, control serum samples (for glucose: ERBA NORM, catalog no. BLT00080, ERBA Diagnostics Mannheim GmbH; for fatty acids and BHB: BOV ASY CONTROL 2, product code AN 1026, Randox Laboratories Ltd.) were run routinely.

The GTT was carried out at around 1000 h after collection of AT samples (see below). Cows were deprived from feed approximately 1 h before and during the GTT. A catheter was inserted into the jugular vein and fixed to the skin 30 min before the test. The catheter was filled with saline dilution of Li-heparin until the start and between blood samplings to avoid clotting. After infusion of a 0.15 g/kg of BW glucose bolus (40% solution), the tubing and catheter were flushed with normal saline. Discarding the first portion, blood samples were collected at the following time points: −5 (basal), 5, 10, 20, 30, 40, 50, and 60 min relative to the start of infusion. Plasma was separated and stored at −80°C until analyzed for glucose as described above, and insulin was analyzed using RIA as described earlier by Vicari et al. (2008). The intra- and interassay CV for insulin were both <10%. The following characteristics were calculated to describe glucose and insulin responses to the GTT: increment (mg/dL), area under the curve 5–20, 5–30, and 5–60 min (mg/dL × min) and reversion time (time to reach basal concentration, T_{basal}; min) for glucose and insulin; clearance rate (CR; %/min) and half-time ($T_{1/2}$; min) for glucose. Increment was calculated as the difference between the maximum and basal concentration. The areas under the curve describe incremental area (Cardoso et al., 2011) and were calculated as the definite integral of the fifth-order polynomial. For the calculation of CR, $T_{1/2}$ and T_{basal} exponential curves were fitted. Following formulas, described by Kaneko et al. (2008), where x is the concentration of glucose corrected for baseline concentration at a given time point and t is time, were used:

$$CR = \frac{\ln(x_{t10}) - \ln(x_{t40})}{t_{10} - t_{40}} \times 100,$$

$$T_{\frac{1}{2}} = \frac{\ln(2)}{CR} \times 100.$$

For the calculation of T_{basal}, the following formula, where x is the concentration of glucose or insulin at a given time point, was used:

$$T_{basal} = \frac{t_{40} - t_{10}}{\ln(x_{t10}) - \ln(x_{t40})} \times 100.$$

Adipose Tissue Biopsies

Subcutaneous AT biopsies were taken after blood sampling for metabolic profiling and before the GTT on d −21 ± 0.6 and d 21 ± 0.2 at around 1000 h from alternate sides of the body from the pin bone region after local anesthesia with lidocaine hydrochloride (Lidocaine–Grindex 20 mg/mL injection solution) under aseptic conditions. Ten minutes after injection of lidocaine hydrochloride, a 2-cm skin incision was made and a tissue sample of about 3 g was collected. Samples were flushed with sterile physiological saline to remove excess of blood and were split into 0.5-g portions onto a sterile Petri dish, mounted into screw-cap tubes (Axygen, Corning Inc., Corning, NY), immediately frozen in liquid nitrogen, and stored at −80°C until analysis. The skin incision was closed with stitches and the wound was covered with an oxytetracycline spray for disinfection and an aluminum spray for mechanical protection.

Processing of Adipose Tissue and Measuring INSR and GLUT4 mRNA Expression

Adipose tissue (100 mg) disruption and homogenization was achieved using the FastPrep-24 (116004500 MP Biomedicals, Illkirch-Graffenstaden, France) for 30 s at 6.5 m/s with a Metal Bead Lysing Matrix (catalog no. 6925–050) in 1 mL of QIAzol Lysis Reagent (catalog no. 79306, Qiagen GmbH, Hilden, Germany). Total RNA was isolated from homogenized tissue with the RNeasy Lipid Tissue Mini Kit (catalog no. 74804, Qiagen GmbH) following the manufacturer's recommendations. Isolated total RNA integrity was determined using a 2100 Bioanalyzer (Agilent Technologies, Massy, France) and RNA 6000 Nano kit (Agilent Technologies Inc., Santa Clara, CA); RNA integrity was found to range between 6.7 and 8.0. The RNA concentrations were determined with the Qubit RNA HS Assay Kit (catalog no. Q32852). Total RNA extracted from tissues was stored at −80°C. For cDNA synthesis, 100 to 250 ng of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (catalog no. 205311, Qiagen GmbH) following the manufacturer's instructions; synthesized cDNA was stored at −20°C. Sample cDNA was used for multiplex quantitative real-time PCR using one 384-well plate for each target gene together with reference gene in 4 replicates. Quantitative real-time PCR was performed using the TaqMan Gene Expression Master Mix (catalog no. 4369016, Thermo Fisher Scientific Inc., Waltham, MA) with the

ViiA 7 Real-Time PCR System (Applied Biosystems, Waltham, MA). Primers and probes were purchased from Applied Biosystems.

GADPH (forward primer sequence: GGCGTGAACCACGAGAAGTATAA, reverse primer sequence: CCCTCCACGATGCCAAAGT, probe sequence: ATACCCTCAAGATTGTCAGCAATGCCTCCT) was used as a reference gene for normalization of mRNA expression levels of the *INSR* (forward primer sequence: TCCTCAAGGAGCTGGAGGAGT, reverse primer sequence: GCTGCTGTCACATTCCCCA, probe sequence: ATCATCTTCAGGCCCTGTTGCCGAG) and *SLC24A* (*GLUT4*; Assay ID: Bt03215323_m1, Thermo Fisher Scientific Inc.) genes. Cycle threshold (**Ct**) values were used to calculate the relative gene expression level using the ΔCt method. For statistical analysis, 4-replicate means of Ct values were used; for 3 samples, regarding *INSR*, and for 11 samples, regarding *SLC24A* (*GLUT4*), 3-replicate means were used due to undetected levels of mRNA.

Processing of Adipose Tissue and Measuring INSR and GLUT4 Protein Expression

Approximately 20 to 200 mg of lipid tissue biopsy was homogenized using the FastPrep-24 (116004500 MP Biomedicals, France) and Lysing Matrix D 2-mL tubes (catalog no. 116913100, MP Biomedicals, LLC, Santa Ana, CA). Prior to homogenizing, the tissue was washed in 1 mL PBS and transferred to a homogenizing tube containing 1 mL of homogenizing solution. The homogenizing solution was based on PBS with a proteinase inhibitor (Complete Ultra Tablets, catalog no. 05892791001, Roche Diagnostics, Indianapolis, IN). The FastPrep-24 Instrument program was as follows: 2 × 30 min with a speed of 6.5 m/s. Dry ice was used for cooling during homogenization. The homogenate was transferred into a new tube and centrifuged at room temperature at 1,000 × *g* for 10 min. The clear homogenate below the upper fat layer was transferred into a new tube. The clear homogenate was used for total protein and target protein concentration measurements. Concentration of target protein was normalized to total protein concentration. The total protein concentration was measured applying the Modified Lowry Protein Assay Kit (catalog no. 23240, Thermo Fischer Scientific Inc., Waltham, MA) according to the manufacturer's protocol in 3 replicates. An amount of 50 to 150 μg of total protein was used for each ELISA reaction. The samples were measured in 2 replicates and means were used for data analysis. For ELISA, commercial kits were used according to the manufacturer's protocol (INSR: catalog no. E11A0445, GLUT4: catalog no. E11G0201, Antibodies-Online Inc., Dunwoody, GA). Inter- and intra-assay CV, provided by the kit's manufacturer, were 7.9 and 5.6% both for INSR and GLUT4.

Statistical Analysis

Before statistical analysis, the continuous energy balance and milk production data were condensed to means on d 21 and up to d 21 and the relative concentrations of *INSR* and *GLUT4* mRNA were multiplied by 1,000 and 100, respectively, to avoid negative values after logarithmic transformation. Statistical analyses were performed with the software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria). To estimate the effect of BCS group and time period on the study variables, the mixed linear model was fitted, considering the fixed effects of BCS group, time, and their interaction, parity, presence of health disorders, relative breeding value for milk production as covariate and random effects of block and cow nested to block. The latter 2 effects allow for consideration of the potential similarity of cows in the same triplet, and repeated measurements of the same cow before and after parturition. For variables measured only after parturition and for GTT characteristics after parturition, the effects of time, interaction between time and BCS group, and cow were excluded from the model. For GTT characteristics before parturition, the presence of health disorders was further excluded. The modeling was performed with the function "lmer" in the package "lme4" and the least squares means (**LSM**, *alias* marginal or model-based means) were estimated with the function "lsmeans." The pairwise comparison of LSM in BCS groups was performed with the function "contrast," and *P*-values were adjusted for multiple testing with the Tukey method. For nonnormally distributed variables, 2 analyses were performed: at first the models were fitted and the LSM were estimated on an actual scale and subsequently the *P*-values were estimated fitting the same models on logarithm-transformed variables. Statistical significance was declared at $P \leq 0.05$ and trend at $P \leq 0.1$.

RESULTS

Health Events, BCS, Energy Balance, and Milk Production

All cows were clinically healthy during the prepartum experimental period. Postpartum, up to d 21 the following health events occurred: dystocia (1 case in group T, 2 cases in group OC), retained placenta (1 case in group OC), metritis (1 case in groups O and OC), mastitis (3 cases in group T, 2 cases in groups O and OC), lameness (2 cases in group O, 1 case in

group OC), and hypocalcemia (1 case in groups T, O, and OC).

The BCS, NEB, and milk production characteristics of experimental groups are presented in Table 2. The BCS differed between all groups on d −21 ($P < 0.001$) and d 21 (T vs. O, $P = 0.006$; T vs. OC, $P = 0.001$; O vs. OC, $P = 0.026$), being lowest in group T and highest in group OC at both selected time points. The OC cows also showed greater body condition loss compared with group T and O cows ($P < 0.001$, Table 2). Among calculated NEB characteristics, energy balance on d 21 and energy balance from parturition to d 21 per day were more negative, and the total deficit from parturition to d 21 was biggest, in group OC compared with groups T ($P < 0.001$; $P = 0.001$; $P < 0.001$, respectively) and O ($P = 0.011$; $P = 0.023$; $P = 0.020$, respectively). The depth of the NEB nadir tended to differ between the group T with the least and group OC with the deepest nadir ($P = 0.058$, Table 2). Differences between the groups' ECM and milk yields on d 21 and yields per day were not significant. Milk fat content on d 21 differed between the group OC, with the highest, and group T, with the lowest, fat contents ($P = 0.031$), whereas milk fat yields between these groups tended to differ ($P = 0.052$). Mean milk fat content during the first 21 d of lactation was highest in group OC, differing from group O with intermediate ($P = 0.019$) and group T with the lowest fat contents ($P = 0.003$). None of the measured and calculated characteristics for milk protein and lactose content or milk production were different between the groups (Table 2).

Glucose Tolerance Test and Blood Metabolite Concentrations

Response curves for glucose and insulin during the GTT are presented in Figure 1, and calculated GTT characteristics and measured blood metabolite concentrations are given in Table 3. The basal concentrations of blood glucose before glucose infusion on d −21 did not differ between the groups (T, 92.7 ± 2.05 mg/dL; O, 95.7 ± 1.98 mg/dL; OC, 96.6 ± 2.05 mg/dL). After glucose infusion, blood glucose concentration increased rapidly, becoming maximum at 5 min in all groups. The maximum was lowest in group T (228.8 ± 7.20 mg/dL), which tended to differ from group O with an intermediate level (251.3 ± 6.96 mg/dL, $P = 0.085$) and differed from group OC with the highest maximum (259.7 ± 7.20 mg/dL, $P = 0.019$). All of the groups differed at 10 min (T vs. O, $P = 0.013$; T vs. OC, $P < 0.001$; O vs. OC, $P = 0.033$); the concentration was still the lowest in group T (200.9 ± 2.97), intermediate in group O (213.8 ± 2.87), and the highest in group OC (225.1 ± 2.97). At 20 min postinfusion glucose concentration in group T remained the lowest (167.9 ± 3.15 mg/dL) and differed from group O with the intermediate level (179.0 ± 3.04 mg/dL, $P = 0.049$) and from group OC with the highest level (184.6 ± 3.15 mg/dL, $P = 0.004$). From 20 min onward, differences between the groups disappeared, the blood glucose concentration continually decreased in all groups. However, at 60 min the preinfusion level had still not been reached (Figure 1). Among calculated GTT characteristics for glucose, the lowest increment in group T was different from the highest increment in group OC ($P = 0.022$). The area under the curve (**AUC**) of glucose was the smallest in group T and largest in group OC. Group O had an intermediate value (Table 3); AUC of glucose 5–20 min was different between all groups (T vs. O, $P = 0.012$; T vs. OC. $P < 0.001$; O vs. OC, $P = 0.029$), AUC 5–30 min in group T was different from group O ($P = 0.028$) and OC ($P < 0.001$), and AUC 5–60 min did not differ between groups (Table 3).

The amplitude of glucose response following glucose infusion on d 21 postpartum was less pronounced compared with d −21 prepartum (Figure 1, Table 3). Starting from the basal level (T, 84.9 ± 3.27 mg/dL; O, 87.6 ± 3.24 mg/dL; OC, 85.4 ± 3.28 mg/dL), blood glucose concentration increased to a maximum at 5 min in all groups (T, 200.9 ± 3.68 mg/dL; O, 206.6 ± 3.65 mg/dL; OC, 212.5 ± 3.69 mg/dL) and from this point onward gradually decreased (Figure 1). No significant differences were observed between the groups for blood glucose concentrations at any time point (Figure 1). Among calculated GTT characteristics highest glucose increment in group OC differed from lowest increment in group T ($P = 0.007$); no significant differences were found between the groups for other calculated GTT characteristics (Table 3).

Blood insulin pre-infusion basal concentration on d −21 was the lowest in group T (13.9 ± 2.32 μU/mL), different from group OC, which had the highest blood insulin level (24.3 ± 2.32 μU/mL, $P = 0.008$). Group O, with an intermediate insulin basal level (17.7 ± 2.25 μU/mL), was not different from the other groups. The subsequent increase of blood insulin was modest in group T. The maximum level (169.8 ± 33.3 μU/mL) in this group was achieved at 10 min followed by a decrease from that time point onward. In groups O and OC blood insulin concentration continued to increase up to 20 min postinfusion, giving rise to differences at this time point between group T, with the lowest (167.4 ± 33.9 μU/mL), and group OC, with the highest insulin level (317.2 ± 33.9 μU/mL, $P = 0.026$). Differences between group O and other groups were not significant. Subsequently, in all groups, blood insulin concentration gradually decreased, whereas differences between group T with the lowest insulin concentrations and group OC

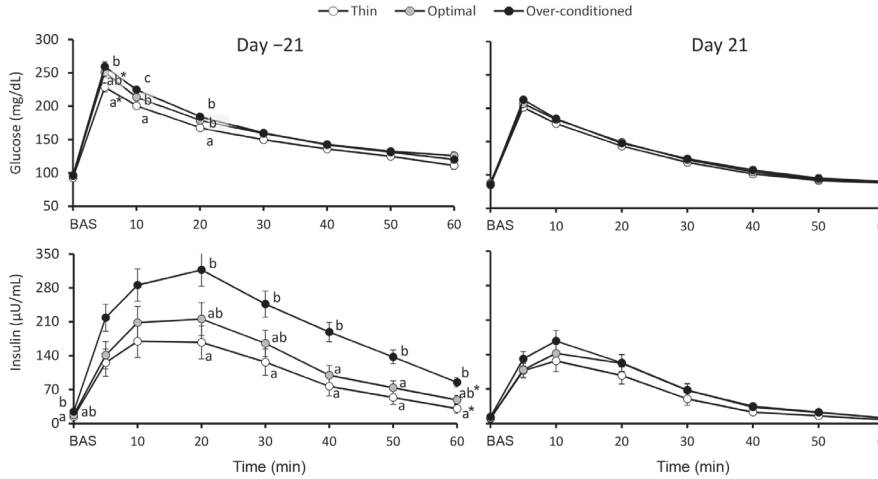

Figure 1. Basal concentrations (BAS) and dynamics for blood glucose (upper panels) and insulin (lower panels) during the i.v. glucose tolerance test (GTT) 21 d before (d −21, left panel) and 21 d after calving (d 21, right panel) in multiparous Holstein dairy cows (n = 42, 14 in each group) grouped according to BCS 4 wk before calving as follows: BCS ≤3.0 (thin; T); BCS = 3.25–3.5 (optimal; O); and BCS ≥3.75 (overconditioned; OC). Different letters (a–c) indicate significant differences between the groups ($P \leq 0.05$) at certain time points, means with asterisks (*) indicate a tendency to differ ($P \leq 0.1$). Error bars represent SEM.

with the highest concentrations persisted up to 60 min postinfusion. In addition, group O differed from group OC at 40 min (99.8 ± 19.4 vs. 188.9 ± 20.1 µU/mL, $P = 0.018$), at 50 min (73.7 ± 13.8 vs. 137.6 ± 14.3 µU/mL, $P = 0.020$) and tended to differ at 60 min postinfusion. At 60 min the pre-infusion level of blood insulin had still not been achieved, being lowest in group T (32.2 ± 6.9 µU/mL), differing from group OC with the highest insulin concentration (82.9 ± 6.9 µU/mL, $P = 0.001$), and tending to differ from group O with the intermediate insulin concentration (51.9 ± 6.9 µU/mL, $P = 0.070$, Figure 1). Regarding calculated GTT characteristics, the lowest increment of blood insulin in group T differed from group OC with the highest increment ($P = 0.031$). The smallest AUC 5–60 in group T differed from the largest AUC in group OC ($P = 0.021$), and reversion time (time to reach basal concentration) in group OC was longer compared with both group T ($P = 0.005$) and group O ($P = 0.011$, Table 3).

Pre-infusion blood insulin basal concentration (T, 9.6 ± 2.26 µU/mL; O, 10.5 ± 2.24 µU/mL; OC, 13.5 ± 2.27 µU/mL) was lower, and the extent of insulin response during the GTT on d 21, was less pronounced compared with d −21 in all groups. The peak of insulin appeared at 10 min in all groups (T, 127.0 ± 21.3 µU/mL; O, 142.6 ± 21.1 µU/mL; OC, 167.6 ± 21.3 µU/mL) followed by a continuous decrease and stabilization at the basal level at 60 min postinfusion (T, 7.6 ± 1.67 µU/mL; O, 10.8 ± 1.65 µU/mL; OC, 11.3 ± 1.67 µU/mL; Figure 1). No significant differences between the groups' blood insulin levels were found at any time point during the GTT, though groups T and O tended to differ at 40 min ($P = 0.092$) and 50 min ($P = 0.087$), or between any of the groups' calculated GTT characteristics (Figure 1, Table 3).

Blood fatty acid concentrations on d −21 did not differ between the groups (T, 0.11 ± 0.08 mmol/L; O, 0.07 ± 0.08 mmol/L; OC, 0.16 ± 0.08 mmol/L). Compared with d −21, fatty acids were higher on d 21 in all groups ($P < 0.001$) and differed ($P = 0.027$) between the groups T with lowest (0.34 ± 0.08 mmol/L) and OC with highest (0.62 ± 0.08 mmol/L) concentration. Group O with intermediate fatty acid concentration (0.47 ± 0.08 mmol/L) did not differ from other groups. Blood BHB concentration did not differ between the groups on d −21 (T, 0.62 ± 0.12 mmol/L; O, 0.61 ± 0.12 mmol/L; OC, 0.55 ± 0.12 mmol/L) or on d 21 (T, 0.56 ± 0.12 mmol/L; O, 0.67 ± 0.12 mmol/L;

Table 3. Least squares means with pooled SEM for blood metabolites before the adipose tissue (AT) biopsy and glucose tolerance test (GTT) and for calculated GTT characteristics in experimental groups 21 d before (d −21) and after calving (d 21)

	d −21					d 21				
	Group					Group				
Characteristic	Thin	Optimal	Overconditioned	SEM	*P*-value	Thin	Optimal	Overconditioned	SEM	*P*-value
Glucose										
Before AT biopsy and GTT (mg/dL)	86.5[a]	90.1[ab]	92.9[b]	2.23	0.016	81.0	82.9	82.0	3.70	0.908
Basal concentration at −5 min (mg/dL)	92.7	95.7	96.6	2.05	0.363	84.9	87.6	85.4	3.28	0.800
Increment (mg/dL)	139*[a]	156[ab]*	163[b]	6.33	0.016	116[a]	119[ab]*	127[b]*	3.03	0.007
Area under the curve 5–20 (mg/dL × min)	1,512[a]	1,656[b]	1,783[c]	33.9	0.000	1,257	1,302	1,346	39.9	0.169
Area under the curve 5–30 (mg/dL × min)	2,169[a]	2,387[b]	2,533[b]	57.8	0.000	1,713	1,776	1,840	63.0	0.287
Area under the curve 5–60 (mg/dL × min)	3,324	3,682	3,770	150	0.095	2,114	2,161	2,348	132	0.383
Clearance rate (%/min)	3.19	3.22	3.60	0.26	0.407	6.08	6.74	5.70	0.73	0.622
Half-time (min)	23.0	23.2	20.6	1.47	0.365	12.2	12.4	14.0	1.31	0.565
Time to reach basal concentration (min)	77.8*	76.4	67.0*	3.49	0.047	55.3	55.8	56.9	3.85	0.955
Insulin										
Before AT biopsy and GTT (μU/mL)	19.9	24.0	24.9	2.98	0.306	12.8	12.3	13.5	2.69	0.819
Basal concentration at −5 min (μU/mL)	13.9[a]	17.7[ab]	24.3[b]	2.32	0.004	9.63	10.7	13.5	2.26	0.718
Increment (μU/mL)	166[a]	208[ab]	310[b]	33.8	0.022	119	133	154	21.5	0.738
Area under the curve 5–20 (μU/mL × min)	2,306*	2,852	4,034*	478	0.072	1,624	1,874	2,092	291	0.644
Area under the curve 5–30 (μU/mL × min)	3,649*	4,603	6,649*	763	0.044	2,256	2,711	2,892	427	0.544
Area under the curve 5–60 (μU/mL × min)	5,308[a]	6,849[ab]	10,867[b]	1,215	0.005	2,616	3,336	3,404	542	0.425
Time to reach basal concentration (min)	45.2[a]	51.7[a]	112.5[b]	14.1	0.001	17.7	22.4	21.5	3.30	0.242

[a–c]LSM with different letters within a row differ ($P \leq 0.05$).
*LSM with an asterisk within a row tend to differ ($P \leq 0.1$).

OC, 0.97 ± 0.12 mmol/L); however, in group OC BHB concentration was higher on d 21 compared with d −21 ($P = 0.045$).

Expression of INSR and GLUT4

Data for AT INSR and GLUT4 expression in the experimental groups are presented in Figure 2. No significant differences in AT *INSR* mRNA abundances between the groups were evident at d −21 or 21. However, *INSR* mRNA abundance on d 21 was higher compared with d −21 in group T (3.2 ± 0.44; 5.9 ± 0.44; $P < 0.001$) and in group O (3.1 ± 0.44; 5.1 ± 0.44; $P = 0.001$). The increase of mRNA from d −21 to 21 in group OC was close to a tendency (3.7 ± 0.45; 4.7 ± 0.45; $P = 0.112$). The extent of adipose INSR protein expression on d −21 was highest in group T (7.3 ± 0.73 ng/mL), different from group O, which had the lowest degree of protein expression (4.6 ± 0.73 ng/mL; $P = 0.040$). Group OC, with an intermediate protein level (6.5 ± 0.74 ng/mL), was not different from either of the other groups. Amounts of AT INSR protein did not change from d −21 to 21 in any of the groups, and significant differences between the groups disappeared on d 21 (Figure 2).

Abundance of AT *GLUT4* mRNA on d −21 tended to differ ($P = 0.075$) between group T (3.5 ± 0.35), with highest level of mRNA expression, and group O (2.4 ± 0.35) with the lowest level, whereas group OC (2.9 ± 0.35) with intermediate mRNA abundance was not different from the other groups. Compared with d −21 AT *GLUT4* mRNA abundance on d 21 remained largely unchanged in group O, whereas a decrease of mRNA occurred in group T ($P = 0.123$) and OC ($P = 0.104$), being reflected in a general time effect ($P = 0.036$; Figure 2). The amount of AT GLUT4 protein on d −21 was lowest in group OC (1.7 ± 0.14 ng/mL), different from group O (1.8 ± 0.14 ng/mL), which had the highest degree of protein expression ($P = 0.002$), and from group T (1.5 ± 0.14 ng/mL), which had an intermediate protein level ($P = 0.024$). From d −21 to 21 GLUT4 protein amount in the AT of cows from

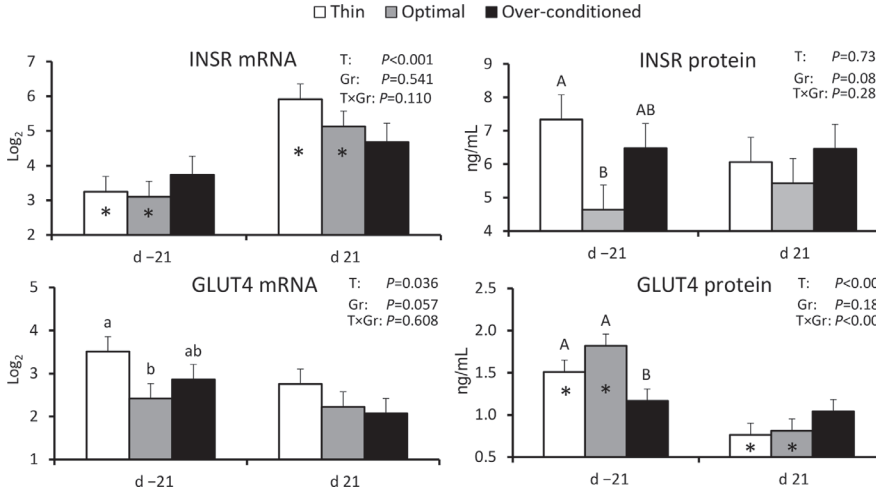

Figure 2. Adipose tissue mRNA and protein expression of insulin receptor (INSR, upper panels) and glucose transporter 4 (GLUT4, lower panels) 21 d before (d −21) and 21 d after calving (d 21) in multiparous Holstein dairy cows (n = 42, 14 in each group) grouped according to BCS 4 wk before calving as follows: BCS ≤3.0 (thin); BCS = 3.25–3.5 (optimal); and BCS ≥3.75 (overconditioned). Abundance of mRNA is expressed as delta cycle threshold (ΔCT) values (2ΔCT); amount of proteins is presented as nanograms per milliliter, measured in homogenate after homogenization and centrifugation of samples. Mean values with different capital letters (A,B) indicate significant differences ($P \leq 0.05$), and means with lowercase letters (a,b) represent a tendency to differ ($P \leq 0.1$). Asterisks (*) indicate significant changes over the time within the group. *P*-values given on the upper-right corner of the graphs refer to time (T) and group (Gr) effects and their interaction (T × Gr). Error bars represent SEM.

group OC did not change, but there was a clear decrease in protein levels in groups T and O ($P < 0.001$), representing a general time effect ($P < 0.001$; Figure 2); significant differences between the groups disappeared on d 21 (Figure 2).

DISCUSSION

Glucose Tolerance Test

In our study we examined the effect of dry period body condition on the development and extent of IR during the transition period. However, the limitation of the study is missing intake data; therefore, we are unable to ascertain that observed changes and differences due to body condition have not been confounded by potential variation of cows DMI. General dynamics of blood glucose and insulin concentrations during the GTT in this study were similar in all experimental groups on both d −21 and 21. However, in all groups the magnitude of responses on d 21 was reduced compared with d −21. The increased clearance for glucose and reduced response for insulin in early lactation compared with late pregnancy is understood to be linked with the physiological homeorhetic adaptation to lactation (Debras et al., 1989; Mann et al., 2016a) and can be explained by greater insulin-independent glucose uptake by the udder and reduced insulin secretion during lactation (Bossaert et al., 2008). In addition, the elevated fatty acid concentrations in all groups of the present study on d 21 compared with d −21 could also have suppressed insulin secretion, as fatty acids may adversely influence pancreatic β-cell functions (Lewis et al., 2002; Stumvoll et al., 2005; Salin et al., 2012). However, when interpreting GTT results, it should be taken into account that glucose and insulin dynamics during the GTT characterize whole-body-level physiological responses and, due to the different physiological status of dry and lactating cows, are not comparable one-to-one (DeKoster et al., 2017).

It is commonly accepted that the acquisition of adequate lipid stores during the dry period plays an important role in successful lactation and balanced metabolic status of cows (Drackley, 1999; Roche et al., 2013a). It is recommended that cows calve at an approximate BCS of 3.25 to 3.5 (Roche et al., 2009) and that cows do not lose or gain BCS during the dry period (Garnsworthy, 2006). Suboptimal BCS and overcondition at calving indicates inappropriate feeding during late lactation and the dry period, and is associated with the development of IR (Hove, 1978; Holtenius et al., 2003; Hayirli, 2006). In this study we observed the lowest blood insulin concentration on d −21 in group T, whereas group OC cows showed the highest insulin concentrations. Concomitantly we observed the lowest responses, both for blood glucose and insulin, during the GTT in group T, intermediate in group O, and the highest responses in group OC. Similarly, Holtenius et al. (2003) reported higher blood insulin concentration and more pronounced insulin responses during the GTT prepartum in overconditioned cows compared with those with optimal body condition and thin cows. As concluded (Holtenius et al., 2003), this was probably due to higher gastrointestinal uptake of insulinotropic precursors such as propionate and glycogenic AA, and thus a higher hepatic rate of glucose synthesis. Highest basal blood insulin concentration and a higher insulin response found in the current study during the GTT in group OC could also be related to obesity-induced β-cell proliferation. An increase in β-cell mass and insulin secretion has been reported in obese mice and humans (Linnemann et al., 2014). According to previous studies, malnutrition with the concurrently suboptimal BCS is supposed to be associated with decreased pancreatic insulin secretion ability (Hove, 1978; Hayirli, 2006; Oikawa and Oetzel, 2006). In the present study, from drying off until switched to the experiment, all cows maintained their BCS, which was the criterion for assignment of experimental groups. Thus, group T cows were thin already at drying off, which may have led to diminished pancreatic insulin secretion ability, being reflected in the lowest insulin response on d −21 in group T. Due to the highest insulin response in group OC, the quickest glucose removal might also have been expected in this group. However, in the present study we observed the highest increment and largest AUC in OC cows, indicating slower removal of circulating glucose and consequently a higher degree of IR compared with the other groups. As proposed by Zachut et al. (2013), a greater insulin response to clear the same dose of glucose indicates the degree of IR. In addition, according to DeKoster et al. (2016b) of the characteristics derived from the i.v. GTT, AUC demonstrates good correlation with the results of hyperinsulinemic euglycemic clamp as a gold standard for assessment of degree of IR, in agreement with our finding of a more pronounced IR in OC cows. Contrary to our findings, probably due to differences in experimental design and conditions, different management and feeding during previous lactation, and different genetic background of cows, no effects of dry period feeding intensity or amount of body fat reserves on glucose and insulin responses before parturition were previously found in several studies (Schoenberg and Overton, 2011; Schoenberg et al., 2012; Mann et al., 2016a).

On d 21 the highest glucose increment in group OC differed from the lowest increment in group T. No other significant differences were present between the groups

on d 21 in blood glucose or insulin responses during the GTT. Similar results have been obtained previously (Mann et al., 2016a). This could be explained by greater insulin-independent glucose consumption for milk synthesis, which can comprise up to 85% of all glucose available in the bloodstream (Knight et al., 1994; Zhao et al., 1996; Etherton and Bauman, 1998). Assuming constant milk synthesis throughout the day, glucose removal by the udder during the GTT exceeded the amount that was infused intravenously. Therefore, we speculate that observed responses for glucose and insulin in the current study in the main reflect the balance between constant insulin-independent removal of glucose and its short-term intravenous infusion. The proportion of insulin-dependent glucose consumption by peripheral tissues is likely small, and differences between the groups, if existent, might be overshadowed by the insulin-independent glucose removal and were not detected. However, Holtenius et al. (2003) reported reduced postpartum glucose disappearance during the GTT in overconditioned cows and attributed this to a more pronounced IR in obese cows. The highest glucose increment in OC cows, observed in the present study, could support this statement.

Expression of INSR and GLUT4

To assess insulin signaling efficiency and potential glucose consumption capability in AT, we measured abundance of mRNA and amount of proteins for INSR and GLUT4. As shown in earlier studies, the development of AT IR is associated with a decrease in the number and binding affinity of INSR on the surface of adipocytes in ewes (Guesnet et al., 1991), humans (Pessin and Saltiel, 2000), and nonruminant species (Flores-Riveros et al., 1993; Brennan et al., 2004; Suagee et al., 2011). Interestingly, in the current study, the abundance of AT *INSR* mRNA was higher on d 21 compared with d −21 independent of BCS, which might indicate increased postpartum insulin signaling capacity in AT compared with prepartum. These results are in agreement with Gross et al. (2011), reporting higher hepatic *INSR* mRNA abundance on wk 1 postpartum compared with wk 3 prepartum and during mid lactation, in cows with feed restriction-induced NEB compared with adequately fed controls. These findings were presumably due to low plasma insulin concentrations, which may have caused an upregulation of *INSR* expression to maintain hepatic insulin function, while maximizing nutrient supply to the mammary gland (Gross et al., 2011). However, in the current study mRNA potential did not lead to increased INSR protein expression on d 21 compared with d −21. Furthermore, despite increased *INSR* mRNA abundance, along with largely unchanged INSR protein expression, we observed a decrease in *GLUT4* mRNA abundance and protein expression on d 21 compared with d −21. As there were no significant differences in AT INSR protein amounts pre- and postpartum, these results support the idea that, in ruminants, insulin signaling in adipocytes is related to signal transmission at the postreceptor, rather than to binding potential of insulin at the receptor level (Vernon and Taylor, 1988; Debras et al., 1989; Sasaki, 2002). It is also possible, that a decrease in GLUT4 expression could be a physiological response to low circulating glucose and insulin concentrations postpartum. Sadri et al. (2010) and Ji et al. (2012) observed downregulation of AT *GLUT4* mRNA expression postpartum compared with prepartum, which is consistent with the present work. However, authors found no differences between the expression of *INSR* mRNA pre- and postpartum (Sadri et al., 2010; Ji et al., 2012). Sadri et al. (2010) concluded that INSR is not strongly involved in the reduction of insulin sensitivity in subcutaneous AT around parturition, whereas the change in insulin-dependent glucose uptake around parturition is regulated in part by GLUT4 presumably being downregulated by elevated fatty acid concentrations, observed also in current study. Ji et al. (2012) suggest that the lack of change in *INSR* mRNA expression around calving, indicating a defect in posttranslational modification of INSR, is likely to be a major mechanism exacerbating IR, whereas the decrease in expression of GLUT4 provided evidence for the reduced responsiveness of AT to insulin during early lactation. Similarly, Wiedemann et al. (2013) suggested that decreased GLUT4 expression contributes to the IR during early lactation more than downregulation of INSR. Mann et al. (2016b) assessed INSR protein concentrations in AT on d 28 prepartum and on d 21 postpartum. In contrast to our results, Mann et al. (2016b) reported a decrease in INSR protein concentration 21 d postpartum compared with 28 d prepartum, being part of the adaptation to lactation and outcome of the reduced concentrations of circulating glucose and insulin.

Regarding AT INSR and GLUT4 expression on d −21, no differences were observed between the *INSR* mRNA abundances of the BCS groups, suggesting an equal capacity for insulin binding and expression of GLUT4 regardless of the amount of body fat stores. Unexpectedly, AT INSR protein concentration, as well as *GLUT4* mRNA abundance on d −21, differed between the group T with the highest and group O with the lowest INSR protein expression and *GLUT4* mRNA abundance. According to Gross et al. (2011) malnutrition-related long-term hypoinsulinemia in thin cows caused an upregulation of the *INSR* expression. As

mentioned earlier, in the present study, group T cows were thin already at drying off, which may have caused an upregulation of the *INSR* expression and consequently promoted transcription of *GLUT4*. In contrast, due to negative feedback, long-term hyperinsulinemia may cause decrease in the quantity of insulin receptors (Suagee et al., 2011) and GLUT4 in AT (Flores-Riveros et al., 1993; Brennan et al., 2004; Suagee et al., 2011). Furthermore, as concluded by Janovick and Drackley (2010) and Janovick et al. (2011), feeding a dairy cow in excess of requirements during gestation, even in the absence of overconditioning, may lead to hyperinsulinemic status. In the present study, blood insulin prepartum basal concentration in groups OC and O exceeded that in group T, which might provide an explanation for reduced INSR protein and *GLUT4* mRNA expression in these cows. The significantly lower GLUT4 protein concentration in group OC compared with groups T and O on d −21, together with the GTT results, is a reflection of the reduced AT capability for glucose uptake in OC cows and provides evidence that in obese cows more pronounced AT IR already develops prepartum, regardless of insulin signaling potential. Similar to these results, no differences were reported shortly prepartum between *INSR* mRNA expression in cows fed the above requirements compared with those fed adequately (Ji et al., 2012). At the same time, Mann et al. (2016b) found a similar INSR protein expression in cows fed during the dry period either according to or above their requirements. In contrast to us, Ji et al. (2012) observed higher prepartum expression of *GLUT4* in overfed cows, possibly due to different sampling time compared with the present study.

Similarly to the GTT results in the present study, no significant differences were observed among BCS groups for AT INSR or GLUT4 expressions on d 21. These results are in accordance with Ji et al. (2012) who reported no differences between *INSR* mRNA and *GLUT4* mRNA expression on d 7 and 21 postpartum in cows fed the above requirements before parturition. Those authors concluded that close-up energy overfeeding did not predispose animals to a more pronounced IR status. Similarly, Mann et al. (2016b) found no differences in INSR protein expression on d 21 between the controlled-energy-fed and overfed cows, leading authors to conclude that overfeeding during the dry period, despite high fatty acid and BHB concentrations postpartum, does not alter the response to a glucose-induced endogenous insulin stimulus in s.c. AT compared with cows fed a controlled diet. However, Zhang et al. (2013) reported that *INSR* mRNA abundance on d 21 postpartum was substantially lower in cows fed a high-energy diet during 21 d prepartum than in cows fed normal or low-energy diets, indicating that the response to insulin in overfed cows was significantly decreased. The present work implies that the amount of body fat reserves during the dry period does not affect AT insulin signaling potential and insulin-dependent glucose consumption capacity at the beginning of lactation. However, this conclusion does not exclude the possibility that the amount of dry period fat stores could influence AT IR in terms of lipolysis and lipogenesis. Indeed, OC cows had the highest plasma fatty acid concentration on d 21 and greatest body condition loss from d −21 to 21, indicating more intensive lipolysis. In addition, compared with groups T and O, cows in the OC group partitioned more fat into milk and had the most negative energy balance, suggesting that overcondition during the dry period is related to intensified lipomobilization at the beginning of lactation. As AT INSR protein amounts on d 21 did not differ between the groups, the presumed inhibition of insulin signaling, along with more pronounced lipolysis in OC cows, probably occurs at the postreceptor signal transduction level.

CONCLUSIONS

The main hypothesis of our study was that the development and extent of IR during the transition period, mediated by the expression and function of INSR and GLUT4, is related to the amount of body fat reserves during the dry period. We demonstrated a considerably lower prepartum AT GLUT4 protein expression in overconditioned cows compared with optimal and thin cows, suggesting a reduced capacity for glucose uptake in the AT of overconditioned cows. This finding, together with the results of the GGT, provides evidence that in obese cows a more pronounced AT IR develops prepartum regardless of similar insulin signaling potential as in the thin and optimal cows, and is related to disturbed GLUT4 protein synthesis. As no significant differences were present between the groups' postpartum INSR and GLUT4 expression, the amount of body fat reserves during the dry period did not affect AT insulin signaling potential or insulin-dependent glucose uptake at the beginning of lactation. Therefore, these results suggest that, regarding glucose metabolism, body condition did not affect AT IR postpartum, but overconditioning during the dry period was related to intensified lipomobilization at the beginning of lactation.

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Body condition and insulin resistance interactions with periparturient gene expression in adipose tissue and lipid metabolism in dairy cows

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ABSTRACT

Adipose tissue plays an important role in a cow's ability to adapt to the metabolic demands of lactation, because of its central involvement in energy metabolism and immunity. High adiposity and adipose tissue resistance to insulin are associated with excessive lipid mobilization. We hypothesized that the response to a glucose challenge differs between cows of different body condition 21 d before and after calving and that the responses are explainable by gene expression in subcutaneous adipose tissue (SAT). In addition, we aimed to investigate insulin resistance with gene expression in SAT and lipid mobilization around parturition. Multiparous Holstein cows were grouped according to body conditions score (BCS) 4 wk before calving, as follows: BCS $\leq$ 3.0 = thin (T, n = 14); BCS 3.25 to 3.5 = optimal (O, n = 14); BCS $\geq$ 3.75 = over-conditioned (OC, n = 14). We collected SAT on d −21 and d 21 relative to calving. A reverse-transcriptase quantitative (RT-q) PCR was used to measure gene expression related to lipid metabolism. One hour after the collection of adipose tissue, an intravenous glucose tolerance test was carried out, with administration of 0.15 g of glucose per kg of body weight (with a 40% glucose solution). Once weekly from the first week before calving to the third week after calving, a blood sample was taken. The transition to lactation was associated with intensified release of energy stored in adipose tissue, a decrease in the lipogenic genes lipoprotein lipase (*LPL*) and diacylglycerol O-acyltransferase 2 (*DGAT2*), and an increase in the lipolytic gene hormone-sensitive lipase (*LIPE*). On d −21, compared with T cows, OC cows had lower mRNA abundance of *LPL* and *DGAT2*, and the latency of fatty acid response after glucose infusion was also longer (8.5 vs. 23.3 min) in OC cows. Cows with higher insulin area under the curve on d −21 had concurrently lower *LPL* and *DGAT2* gene expression and greater concentration of fatty acids on d −7, d 7, and d 14. In conclusion, high adiposity prepartum lowers the whole-body lipid metabolism response to insulin and causes reduced expression of lipogenic genes in SAT 3 weeks before calving. In addition, more pronounced insulin release after glucose infusion on d −21 is related to higher lipid mobilization around calving, indicating an insulin-resistant state, and is associated with lower expression of lipogenic genes in SAT.

Key words: body condition score, insulin resistance, dairy cow, adipose tissue metabolism

INTRODUCTION

Dairy cows' physiological adaption to lactation has been extensively researched in recent years, as its success is crucial for the overall lactation period. Nevertheless, the transition to lactation remains the most critical period in the production cycle of dairy cows, due to the risk of excessive lipid mobilization and of disease. Adipose tissue plays an important role in a cow's ability to adapt to her new physiological state, because of its central involvement in energy metabolism and immunity (Contreras and Sordillo, 2011). The volume of subcutaneous adipose tissue (**SAT**) can be accurately quantified through BCS, and under- or overconditioned cows at calving face greater risks of health problems (Roche et al., 2009). Physiological processes before parturition are assumed to blunt the insulin response in insulin-sensitive tissues, causing insulin resistance (**IR**) during transition to lactation, to support energy and nutrient flow to the mammary gland (De Koster and Opsomer, 2013). Insulin is an important antilipolytic hormone, and therefore its low concentration in plasma, or a blockage in its signal transduction in adipose tissue, causes intensified lipolysis that may result in lipotoxicity due to the high concentration of fatty acids in plasma. It has been argued that IR in adipose tissue facilitates high fatty acid release postpartum

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(Bell, 1995). However, this concept has recently been questioned, as evidence from both clamp (Weber et al., 2016; Grossen-Rösti et al., 2018) and mRNA (Ji et al., 2012) studies have failed to support this idea. On the other hand, a common finding in intravenous glucose tolerance test (**IVGTT**) studies is a slight or no difference in glucose area under the curve (**AUC**), but considerable increase in insulin AUC for over-conditioned compared with adequately conditioned cows (Bogaert et al., 2018; Jaakson et al., 2018) and over-fed cows prepartum (Holtenius et al., 2003), which supports the IR hypothesis. In addition, we previously (Jaakson et al., 2018) demonstrated lower glucose transport protein 4 (GLUT4) concentrations in the adipose tissue of over-conditioned cows 3 weeks before parturition. De Koster et al. (2015) also found decreased insulin sensitivity of glucose metabolism for over-conditioned cows. On the other hand, at physiological concentrations of insulin its antilipolytic effect remained intact (De Koster et al., 2015, 2016). However, cows with excessive adipose tissue at parturition are well known to release more stored lipids (Roche et al., 2009). These findings highlight the complexity of interactions involved in insulin signaling in adipose tissue and the need for additional studies to elucidate the causes of excessive lipid mobilization after parturition.

In the present study, we hypothesized that the response to a glucose challenge differs between cows with different body condition 21 d before and after calving, and these differences are explainable by gene expression in SAT. We aimed to use the IVGTT to assess the effect of insulin on lipid metabolism in relation to the volume of SAT prepartum, and to investigate the expression of genes related to lipolysis and lipogenesis in SAT. In addition, we aimed to associate IR with gene expression in SAT and lipid mobilization around parturition.

MATERIALS AND METHODS

Experimental Design

In this experiment, we followed the European Council Directive regarding the protection of animals and the Estonian Animal Protection Act. The study was approved by the Committee for Conducting Animal Experiments at the Estonian Ministry of Agriculture. Detailed descriptions of the experimental design, animals, feeds, and methods have been reported previously (Jaakson et al. 2018). In brief, the study was carried out on the experimental farm of the Estonian University of Life Sciences (Tartu, Estonia), with a herd size of 120 cows and a mean annual milk yield of 9,200 kg/cow. Over 2 consecutive years (2013 to 2015), 42 indoor-housed multiparous Estonian Holstein cows were assigned to 3 experimental groups on the basis of BCS 28 d before expected calving (d −28) as follows: BCS $\leq$ 3.0 (2.25 to 3.00) = thin (**T**, n = 14); BCS of 3.25 to 3.5 = optimal (**O**, n = 14); BCS $\geq$ 3.75 (3.75 to 4.50) = over-conditioned (**OC**, n = 14). We assessed BCS fortnightly, according to the method described by Edmonson et al. (1989), from drying-off until d −28 prepartum. Cows whose BCS did not change in this period were enrolled, in a blocked design with each block consisting of 3 cows, 1 from each group. After calving, BCS was assessed weekly. The average parity of the cows was as follows: T = 2.6 (9 parity 2, 3 parity 3, 1 parity 4, 1 parity 5); O = 3.2 (5 parity 2, 4 parity 3, 3 parity 4, 1 parity 5, 1 parity 6); OC = 3.7 (1 parity 2, 6 parity 3, 4 parity 4, 2 parity 5, 1 parity 6). On d −28, cows were removed from the dry-cow barn to tiestall housing, to adjust to experimental conditions. After calving, from the seventh milking onward, cows were housed in a freestall barn with a milking parlor. Cows were milked twice daily.

Diets and their ingredients and chemical compositions are presented in Table 1. Cows were fed TMR ad libitum twice daily, at 0530 and 1430 h. Diets were calculated according to Estonian feeding recommendations: ME according to Oll (1995), MP as described by Kärt et al. (2002).

BCS Loss, Blood Sampling, Glucose Tolerance Test, and Laboratory Analyses

To calculate BCS loss, weekly BCS measurements were modeled as described by Thorup et al. (2012). The loss was defined as the total BCS loss during the first 21 DIM.

Blood samples were taken on d −7 (±2.6; mean ± SD), d 7 (±0.9), d 14 (±0.8), and d 21 (±1.0) at approximately 1000 h. Blood was drawn from the coccygeal vein into vacuum tubes containing Li-heparin (Vacuette, Greiner Bio-One International GmbH, Kremsmünster, Austria), centrifuged (5,000 × *g*, 15 min, 4°C), and stored at −80°C. We used an enzymatic-colorimetric method (cat. no. FA 115; Randox Laboratories Ltd., Crumlin, UK) to analyze fatty acids, and BHB was analyzed using the kinetic-enzymatic UV method (cat. no. RB 1007; Randox Laboratories) with an automatic analyzer (ERBA XL300; Randox Laboratories). The inter-assay CV were 8.3% and 6.1%, and intra-assay CV were 6.1% and 5.7% for fatty acids and BHB, respectively.

The IVGTT was carried out on d −21 and on d 21 relative to calving. Relative to the start of jugular vein infusion of a 0.15 g/kg of BW glucose (40%) solution, blood samples were collected at −5 (basal), 5, 10, 20, 30, 40, 50, and 60 min. Blood samples were centrifuged,

Table 1. Diet ingredients and chemical composition (mean ± SD) of TMR diets

Item	Diet				
	Far-off until d −15	Close-up d −14 to −1	Lactation 1 d 1 to 6	Lactation 2 d 7 to 14	Lactation 3 from d 15
Ingredient, g/kg					
Grass silage	955 ± 81	599 ± 71	604 ± 72	460 ± 49	384 ± 37
Hay	33.6 ± 81	28.3 ± 69	28.6 ± 70	20.3 ± 49	15.6 ± 37
Barley meal	—	301 ± 10	303 ± 10	309 ± 0.1	296 ± 0.2
Corn meal	—	—	—	64.3 ± 0.0	120 ± 0.1
Heat-treated rapeseed cake	—	47.1 ± 0.6	47.5 ± 0.6	129 ± 0.0	168 ± 0.1
Mineral-vitamin feed	11.7 ± 0.1[1]	7.82 ± 0.2[1]	10.5 ± 0.3[2]	8.58 ± 0.0[2]	7.99 ± 0.0[2]
Anionic mineral feed[3]	—	10.4 ± 0.2	—	—	—
Limestone	—	6.26 ± 0.2	—	4.29 ± 0.0	4.41 ± 0.7
Sodium chloride	—	—	5.78 ± 0.1	4.72 ± 0.0	4.40 ± 0.0
DM of diet, g/kg	348 ± 78	435 ± 64	433 ± 64	483 ± 53	511 ± 45
Chemical composition					
CP, g/kg of DM	131 ± 10.5	144 ± 7.2	145 ± 7.3	161 ± 5.6	169 ± 4.8
MP, g/kg of DM	72.8 ± 3.3	86.6 ± 2.1	87.3 ± 2.1	97.9 ± 1.6	104 ± 1.3
ME, MJ/kg	8.70 ± 0.3	10.1 ± 0.2	10.2 ± 0.2	10.9 ± 0.1	11.3 ± 0.1
NDF, g/kg of DM	534 ± 51	455 ± 32	458 ± 32	410 ± 25	380 ± 22
ADF, g/kg of DM	387 ± 47	278 ± 29	280 ± 29	242 ± 22	220 ± 18
Ca, g/kg of DM	11.4 ± 1.9	10.3 ± 1.3	9.33 ± 1.3	9.54 ± 1.0	8.97 ± 0.8
P, g/kg of DM	3.42 ± 0.4	4.06 ± 0.2	4.03 ± 0.2	4.48 ± 0.2	4.77 ± 0.2

[1]Composition (as-fed basis): 170 g/kg of Ca; 50 g/kg of P; 30 g/kg of Na; 140 g/kg of Mg; 30 g/kg of S; 1,000 mg/kg of Cu; 4,500 mg/kg of Zn; 4,000 mg/kg of Mn; 40 mg/kg of Se; 50 mg of Co; 200 mg of I; 800,000 IU/kg of vitamin A; 190,000 IU/kg of vitamin D; and 8,000 IU/kg of vitamin E.

[2]Composition (as-fed basis): 150 g/kg of Ca; 35 g/kg of P; 75 g/kg of Na; 90 g/kg of Mg; 1 g/kg of S; 4,000 mg/kg of Cu; 6,667 mg/kg of Zn; 6,452 mg/kg of Mn; 94 mg/kg of Se; 109 mg of Co; 650,000 IU/kg of vitamin A; 150,000 IU/kg of vitamin D; and 4,000 IU/kg of vitamin E.

[3]Composition (as-fed basis): 9 g/kg of Ca; 1 g/kg of P; 5 g/kg of Na; 100 g/kg of Mg; 1,000 mg/kg of Cu; 5,000 mg/kg of Zn; 2,000 mg/kg of Mn; 27 mg/kg of Se; 40 mg of Co; 100 mg of I; 1,000,000 IU/kg of vitamin A; 60,000 IU/kg of vitamin D; 10,000 mg/kg of vitamin E; and 100,000 μg/kg of biotin.

and plasma stored at −80°C until analyzed for fatty acids and BHB, as described above. Decreases of fatty acids and BHB were calculated as the difference between basal and minimum concentrations and expressed as percentage from the basal concentration. Latency was defined as the point at which the second-order polynomial fitted to fatty acid values between minutes 5 and 60, expressed as the percentage of basal fatty acids, crossed the x-axis. If values were lower than the average infusion time of 2 min, then the latency period length was expected to be 2 min. The calculations and values of insulin AUC have been previously reported (Jaakson et al., 2018).

Sampling and Processing of Adipose Tissue

We took SAT biopsies before the IVGTT procedures, from the pin bone region of each cow after local anesthesia [Lidocaine-Grindeks (Riga, Latvia) 20 mg/mL injection solution] under aseptic conditions on d −21 and d 21 relative to calving. A 2-cm skin incision was made, and a tissue sample of about 3 g was collected, flushed with physiological saline, mounted into screw-cup tubes (Axygen, Corning Inc., Corning, NY), immediately frozen in liquid nitrogen, and stored at −80°C. The skin incision was closed with stitches, and the wound was disinfected and covered with an aluminum spray for mechanical protection.

For RNA analyses, adipose tissue was homogenized using the FastPrep-24 (116004500, MP Biomedicals, Illkirch-Graffenstaden, France) with a Metal Bead Lysing Matrix (cat. no. 6925-050; MP Biomedicals) in QIAzol Lysis Reagent (cat. no. 79306, Qiagen GmbH, Hilden, Germany), and total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (cat. no. 74804, Qiagen). We determined RNA integrity using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and RNA 6000 Nano kit (Agilent Technologies), and it was found to range between 6.7 and 8.0. We determined RNA concentrations with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). Total RNA, extracted from tissues, was stored at −80°C until cDNA synthesis using the QuantiTect Reverse Transcription Kit (cat. no. 205311, Qiagen). Synthesized cDNA was stored at −20°C until used for PCR.

Multiplex quantitative real-time PCR was carried out on one 384-well plate for each target gene together with reference gene in 4 replicates using the TaqMan Gene Expression Master Mix (cat. no. 4369016, Thermo Fisher Scientific) with the ViiA 7 Real-Time PCR System

(Applied Biosystems, Thermo Fisher Scientific). All primers and probes were from Thermo Fisher Scientific. We used *GAPDH* (Assay ID: Bt03210913_g1) as a reference gene for normalization of mRNA expression levels of the genes leptin (*LEP*; assay ID: Bt03211909_m1), lipoprotein lipase (*LPL*; assay ID: Bt03240499_m1), solute carrier family 27 member 1 (*SLC27A1*, fatty acid transport protein 1; assay ID: Bt03252013_m1), diacylglycerol O-acyltransferase 2 (*DGAT2*; assay ID: Bt03259839_m1), and hormone-sensitive lipase [*LIPE* (HSL); assay ID: Bt03253691_m1). Cycle threshold (Ct) values were used to calculate relative gene expression levels using the ΔCt method. For statistical analysis, we used the mean of 4 replicates' Ct values.

Statistical Analysis

Before statistical analysis, the relative mRNA concentrations of *LEP* and *SLC27A1* were multiplied by 100; *LIPE*, *LPL*, and *DGAT2* were multiplied by 10, to avoid negative values after logarithmic transformation. Statistical analyses were performed with the software R (version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria). Pearson correlation coefficients were calculated to investigate the relationships between fatty acids and mRNA, between fatty acids and insulin AUC, between mRNA and insulin AUC, and between insulin AUC and BCS loss. To estimate the effect of BCS group and time on the dependent variables, a mixed linear model was fitted, considering the fixed effects of BCS group, time of IVGTT, their interaction, and the confounding factors of parity, relative breeding value for milk production, and presence of health disorders, and random effects of block and cow nested to block. Disorders occurred only in the postpartum period and were dichotomized: (1) no occurrence of disorders or (2) an occurrence of any clinical disorder. The complete list of disorders has been reported earlier (Jaakson et al., 2018). Pre- and postpartum IVGTT data were modeled separately. Thus, the effects of time of IVGTT, time of IVGTT and group interaction, and cow were excluded from the model for postpartum data, and in addition, the presence of health disorders for prepartum data. Modeling was performed with the function lmer in the R package lme4, and the least squares means (LSM, also called marginal or model-based means) were estimated with the function lsmeans. The pairwise comparison of LSM was performed with the function contrast. The Tukey method was used to adjust *P*-values for multiple testing. Two analyses were performed for variables that were not normally distributed: first the models were fitted and LSM were estimated on an arithmetic scale; subsequently *P*-values were estimated, fitting the same models on log-transformed values. Statistical significance was declared at $P < 0.05$ and tendency at $P < 0.1$.

RESULTS

Glucose Tolerance Test

In general, the infusion of glucose, after the initial latency, was associated with a decrease in fatty acid and BHB concentrations before and after calving (Figure 1). The basal concentrations and proportional decrease of fatty acids on d −21 were similar between groups, but, between 10 and 60 min, group T differed from the OC group ($P < 0.05$), and, between 20 and 50 min, group O differed from OC ($P < 0.05$). Latency of fatty acids was the shortest in group T compared with groups O ($P < 0.05$) and OC ($P < 0.01$; Table 2). On d 21, the dynamics were similar between groups (Figure 1), but a difference appeared at 20 and 30 min between groups T and OC ($P < 0.05$).

The concentration of BHB on d −21 in group T, with the highest values throughout the IVGTT, tended to differ from group O at 40 min ($P = 0.08$) and differed from group O ($P < 0.05$) at 50 min. On d 21 the BHB concentration in the OC group only tended to differ from group T ($P = 0.09$) at min 10, where OC was higher (Figure 1). Insulin AUC on d −21 in group T with the lowest value differed from group OC with the highest value ($P < 0.01$).

Gene Expression

Time was a significant factor for all of the studied genes' mRNA abundance ($P < 0.01$, Figure 2). Abundance was higher on d 21 than on d −21 for *LIPE* and *SLC27A1* and lower for *LEP*, *DGAT2*, and *LPL*. We found a group effect for *DGAT2* ($P < 0.05$) and *LPL* ($P < 0.01$) mRNA abundance. On d −21, the OC group had the lowest *DGAT2* and *LPL*, and this group's mRNA abundance differed from group T ($P < 0.05$), which had the lowest expression. For *LPL*, analysis also revealed a tendency to differ between groups T and O ($P = 0.07$). No differences were found between experimental groups on d 21 (Figure 2).

Pearson Correlations

In the calculation of Pearson correlations, the BCS groups were not differentiated. The Pearson correlation coefficients of gene expression and insulin AUC on d −21 with fatty acids on d −7 and d 7 are presented in Figure 3. Insulin AUC on d −21 with gene expressions

Table 2. Characteristics describing the dynamics of metabolites during intravenous glucose tolerance test

	d −21					d 21				
Characteristic	Thin	Optimal	Over-conditioned	SEM	*P*-value	Thin	Optimal	Over-conditioned	SEM	*P*-value
Fatty acids[1]										
Basal, mmol/L	0.18	0.17	0.24	0.02	0.04	0.45	0.68	0.72	0.13	0.18
Latency,[1] min	8.5[a]	16.9[b]	23.3[b]	3.00	<0.01	10.0	7.94	12.9	1.80	0.12
Decrease,[2] %	38.0	34.1	33.7	4.06	0.67	46.5	55.7	43.5	5.14	0.20
BHB										
Basal, mmol/L	0.49	0.44	0.42	0.01	0.24	0.52	0.75	1.06	0.20	0.11
Decrease,[2] %	18.2	23.2	19.5	3.90	0.62	22.7	35.8	31.2	3.89	0.05
AUC,[3] 5–60 min										
Glucose, mg/dL × min	3,324	3,682	3,770	150	0.10	2,114	2,161	2,348	132	0.39
Insulin, µg/dL × min	5,308[a]	6,849[ab]	10,867[b]	1,215	0.01	2,616	3,336	3,404	542	0.43

[a,b]Values within a row with different superscripts are significantly different ($P < 0.05$).

[1]The time from the start of glucose infusion until the concentration dropped below the basal value.

[2]The difference between basal and minimum, expressed as a percentage of the basal value.

[3]Area under the curve.

and BCS loss are presented in Figure 4. The average concentrations of fatty acids were as follows (mean ± SD): d −7, 0.13 ± 0.071 mmol/L; d 7, 0.59 ± 0.362 mmol/L; d 14, 0.49 ± 0.302 mmol/L; d 21, 0.53 ± 0.443 mmol/L. We observed a positive correlation between insulin AUC on d −21 and plasma fatty acids on d −7, d 7 (r = 0.49 and 0.55, respectively; $P < 0.01$; Figure 3), and d 14 (r = 0.39; $P < 0.05$; data not shown). The mRNA abundance of *LPL* on d −21 was negatively correlated with fatty acids on d −7 and d 7 (r = −0.32 and −0.40, respectively; $P < 0.05$), and the mRNA abundance of *DGAT2* on d −21 tended to be negatively correlated with fatty acids on d −7 (r = −0.31; $P = 0.06$) and negatively correlated on d 7 (r =

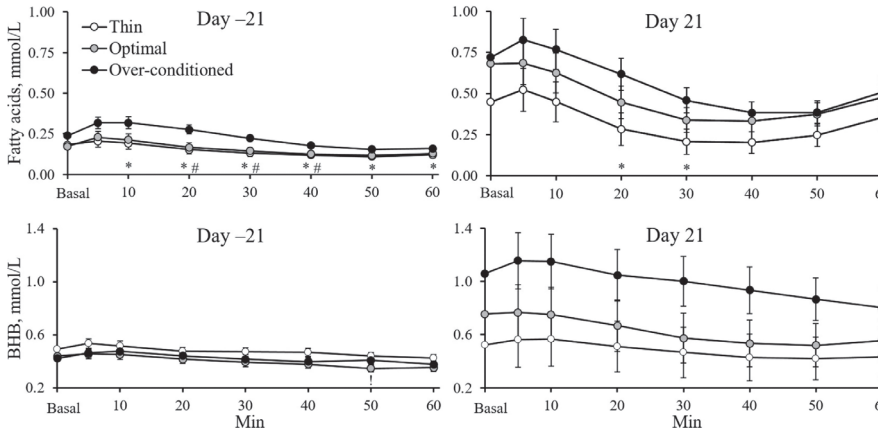

Figure 1. Concentrations of fatty acids and BHB during the i.v. glucose tolerance test on d −21 and 21 relative to calving in multiparous Holstein cows, grouped according to BCS on d −28 as follows: ≤3.0 = Thin; 3.25 to 3.5 = Optimal; ≥3.5 = Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. *Indicates a difference ($P \leq 0.05$) between Thin and Over-conditioned groups; ! indicates difference between Thin and Optimal groups; and # indicates difference between Optimal and Over-conditioned groups. No significant difference was found between Thin and Optimal groups.

−0.30; $P < 0.05$). In addition, insulin AUC on d −21 was negatively correlated with the mRNA abundance of the lipogenic genes *LPL* and *DGAT2* (r = −0.32 and −0.31, respectively; $P < 0.05$) and positively with total BCS loss during 21 DIM (r = 0.48; $P < 0.01$).

DISCUSSION

This paper is a companion paper to that of Jaakson et al. (2018), which investigated glucose metabolism in dairy cattle and reported the indices of glucose and insulin.

IVGTT

Insulin is a potent inhibitor of lipolysis; its signaling reduces the cellular concentration of cyclic AMP (cAMP) that, in turn, reduces protein kinase A (PKA)–mediated lipolysis (Bolsoni-Lopes and Alonso-Vale, 2015). Although lipolysis was low on d −21 (low basal fatty acid concentration), insulin further repressed lipolysis, as we observed a decrease in fatty acids in all groups. We previously reported the highest insulin AUC prepartum in OC cows compared with T and O cows (Jaakson et al., 2018), but this did not result in enhanced fatty acid clearance. On the contrary, OC cows had higher values throughout IVGTT. The insulin peak, even in group T with the smallest insulin AUC (Jaakson et al., 2018), was sufficient for maximal inhibitory effect on lipolysis, according to the insulin-dose response curve calculated by De Koster et al. (2015). This explains the similar fatty acid decrease between groups. The greater fatty acid concentrations during IVGTT in the OC group on d −21 are explainable by the positive correlation between the size of the adipocytes and the basal lipolytic rate (De Koster et al., 2016), which insulin does not control. On the other hand, it could be argued that IR might be responsible for the differences we observed during IVGTT, as high insulin AUC is an indicator of increased pancreatic insulin secretion to compensate for peripheral IR (Bogaert et al., 2018). In support of this, Salin et al. (2018) used latency of the fatty acid response after glucose infusion to assess insulin sensitivity, and latency in our study was associated with BCS group and was lowest in group T. However, little information is available on the cause of the duration of latency period. We assume that the rapid increase of glucose in plasma hinders the use of other energy sources (e.g., fatty acids, BHB), and the latency describes the time it takes for the insulin signaling network to suppress the production and increase the elimination of these substances. To conclude, OC cows had the highest levels of insulin secretion after glucose infusion, greater fatty acid values during IVGTT, and longer latency compared with thinner cows—all of which indicates that high adiposity in the prepartal period lowers the lipid metabolism response to insulin, in insulin-sensitive tissues.

The decreasing BHB concentration during the prepartum IVGTT is a result of increased glucose concentration (suppression of ketogenesis in the liver), decreased fatty acid concentration (decreased availability of substrate), and elimination of BHB from blood. The basal BHB values prepartum were low in all groups, and the dynamics after glucose infusion were similar. Even though a difference appeared at 50 min between

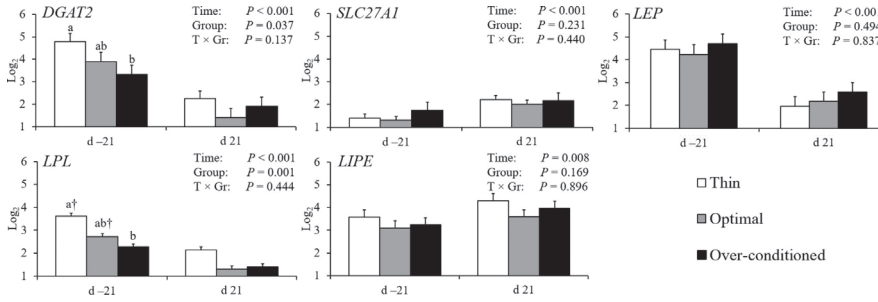

Figure 2. Expression of diacylglycerol O-acyltransferase 2 (*DGAT2*), solute carrier family 27 member 1 (*SLC27A1*), leptin (*LEP*), lipoprotein lipase (*LPL*), and hormone-sensitive lipase (*LIPE*) on d −21 and 21 relative to calving in subcutaneous adipose tissue of multiparous Holstein cows grouped according to BCS on d −28 as follows: ≤3.0 = Thin; 3.25 to 3.5 = Optimal; ≥3.5 = Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Different letters (a, b) indicate difference ($P \leq 0.05$); †indicates tendency to differ ($P \leq 0.1$).

groups T and O during IVGTT, we do not believe it has physiological relevance.

We observed no differences between groups in the dynamics of fatty acids after glucose infusion. This suggests that the volume of SAT does not interfere with insulin antilipolytic signal on d 21, as Weber et al. (2016) also concluded, and we have to agree with Salin et al. (2017) that the response of fatty acids after glucose infusion is driven largely by basal fatty acid concentration.

Effect of Lactational Stage on SAT Gene Expression

Previous studies indicate that lipogenesis in early lactation is mainly controlled at the transcription level, whereas control of lipolysis occurs at a posttranslational level (Khan et al., 2013). We observed significantly lower *DGAT2* and *LPL* mRNA abundance on d 21 compared with d −21. Therefore, potentially lower availability of lipoprotein-derived fatty acids (due to decreased *LPL*) and lower intracellular (re-)esterification of fatty acids to triacylglycerol (due to decreased *DGAT2*) may occur in adipose tissue in early lactation compared with prepartum. Van den Top et al. (2005) have also reported lower LPL protein activity postpartum. In adipose tissue, insulin positively regulates LPL transcription and enzyme activity (Kersten, 2014), whereas energy deficit (Bonnet et al., 2004) regulates them negatively. This means that the physiological state of a dairy cow in the immediate postpartum period favors the downregulation of LPL in adipose tissue and, thus, energy partitioning toward the mammary gland.

The mRNA abundance of *LIPE* has been variously shown to increase (Sumner and McNamara, 2007), not to change (Sadri et al., 2010), or to decrease (Ji et al., 2012; De Koster et al., 2018) in transition from the dry period to lactation. However, the increase in phosphorylation of hormone-sensitive lipase (HSL, the protein of *LIPE*) has been consistently demonstrated (Mann et al., 2016; De Koster et al., 2018). This highlights the importance of post-translational modification of HSL and limits the possibility of drawing associations between lipolysis and mRNA abundance of *LIPE*.

Higher expression of *SLC27A1* postpartum is contrary to the changes we found for the other lipogenic pathway genes (*LPL* and *DGAT2*). This suggests that not all lipogenic genes are downregulated postpartum and that different control mechanisms may exist for the studied genes. Ji et al. (2012) also found an overall decrease in the expression of lipogenic genes postpartum, except for genes related to fatty acid uptake.

The present study showed a decrease in leptin expression postpartum that accords with the findings of Vailati-Riboni et al. (2016). In addition, Kokkonen et al. (2005) and Mann et al. (2018) observed less leptin protein in plasma. It seems that the transcription and

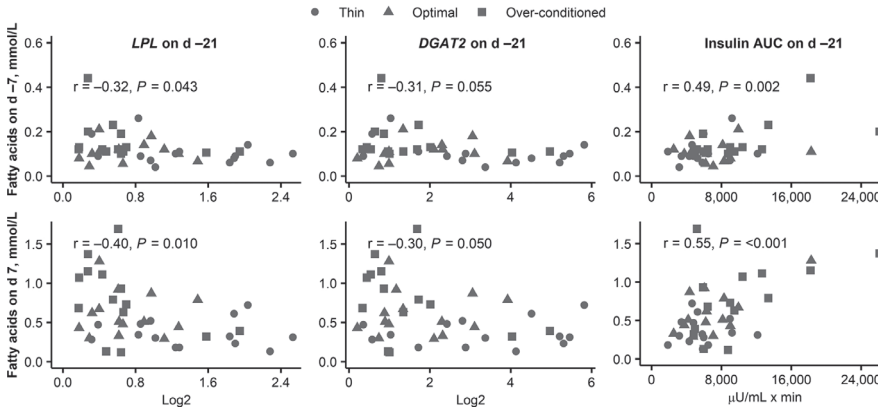

Figure 3. Pearson correlation coefficients (r) of d −21 lipoprotein lipase mRNA (*LPL*), diacylglycerol O-acyltransferase 2 mRNA (*DGAT2*), and insulin area under the curve (AUC) with fatty acids on d −7 and 7 relative to calving. Points represent the values of individual multiparous Holstein cows.

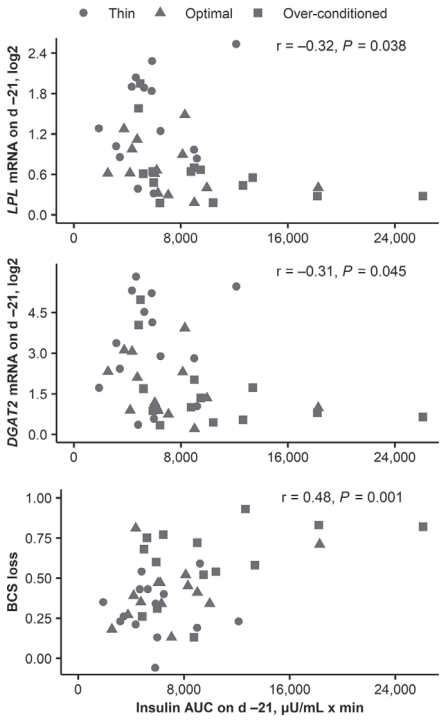

Figure 4. Pearson correlation coefficients (r) of lipoprotein lipase mRNA (*LPL*), diacylglycerol O-acyltransferase 2 mRNA (*DGAT2*), and total BCS loss in the first 21 DIM, with insulin area under the curve (AUC) on d −21. Points represent the values of individual multiparous Holstein cows.

protein expression of leptin are both downregulated during negative energy balance.

Effects of BCS on Gene Expression in SAT

We found reduced expression of the lipogenic genes *LPL* and *DGAT2* for OC cows on d −21, suggesting that high adiposity is associated with decreased lipogenesis in SAT. This supports the results of Alharthi et al. (2018), who found a reduced mRNA abundance of phosphoenolpyruvate carboxykinase 1, a key enzyme for glyceroneogenesis, in SAT of over-conditioned cows prepartum and concluded that reduced re-esterification of fatty acids occurs in those cows. The *LPL* and *DGAT2* expression results may partly explain the differences in fatty acid dynamics in our study after glucose infusion prepartum. Group T cows showed a quicker reduction of fatty acids during IVGTT, which may have been a result of a quicker elimination and re-esterification of plasma fatty acids in adipose tissue.

Regarding the early postpartum period, Alharthi et al. (2018) reported higher expression of genes involved in fatty acid oxidation for over-conditioned cows on d 7 and 20 of lactation, supporting the overall notion of over-conditioned cows using more stored lipids postpartum. This agrees with the greater basal fatty acid and BHB concentrations for OC cows in our study and confirms higher lipid mobilization for OC cows on d 21. Nevertheless, we did not observe any differences in the dynamics of fatty acids and BHB during IVGTT or on the mRNA abundance of genes on d 21, even though the BCS was different between the groups (Jaakson et al., 2018). This suggests that, in the third week of lactation, the volume of SAT is related neither to expression of the studied genes nor to insulin signaling. Therefore, neither the differences in gene expression nor insulin signaling explain the higher lipid mobilization in OC cows.

We observed neither different *LEP* expression between the experimental groups nor correlations between *LEP* and BCS (data not shown) on d −21 and 21, even though leptin is mainly synthesized in AT and its relations to high adiposity (Chilliard et al., 2005) and to adipocyte size (Depreester et al., 2018) have been established. In agreement with our results in the prepartum period, Schoenberg et al. (2011) did not find BCS to be an important factor for *LEP* expression, Mann et al. (2018) reported a weak correlation between plasma leptin and BCS, and Pires et al. (2013) found elevated plasma leptin only in cows that had higher BCS compared with the OC cows in our study. As a small adipose depot (De Koster et al., 2015) the *LEP* expression of SAT is lower than those of other depots (Depreester et al., 2018); therefore the contribution of the expression of *LEP* in SAT, compared to plasma leptin concentration, may be also smaller. Hormonal agents (Block et al., 2003) and energy balance (Singh et al., 2014) also regulate leptin in plasma; thus, different feeds and feeding regimens might explain the discrepancies between studies.

In the postpartum period Holtenius et al. (2003), Mann et al. (2018), and Mansouryar et al. (2018) did not find plasma leptin to be related to BCS. These results agree with our mRNA data and suggest that the volume of SAT has no effect on either the concen-

tration of leptin in plasma or *LEP* expression in SAT postpartum.

Insulin Resistance

Salin et al. (2017) verified the hyperbolic relationship between insulin secretion and insulin sensitivity in dairy cows, which means that a change in sensitivity will be compensated by increased insulin secretion. Therefore, we consider that insulin AUC can be used as the measure for IR.

Endocrine status at the beginning of lactation (low insulin and high growth hormone concentration), in conjunction with adrenergic signaling, favors mobilization of body lipids through increased cellular concentration of cAMP. If insulin-sensitive cells in a dairy cow are resistant to insulin, the main antagonist of adrenergic signaling, then further lipids are mobilized.

We showed that insulin AUC prepartum has a negative correlation with lipogenic gene expression in SAT. Our data also indicate that the physiological state, closely mirrored by IR status on d −21, carries over to the later weeks pre- and first weeks postpartum. Cows with more prominent insulin release 3 wk before parturition tend to use more body lipids in the weeks around calving, when the release of energy stored in adipose tissue is the highest. This conclusion is supported by the positive correlations between fatty acids and BCS loss with insulin AUC. Zachut and Moallem (2017) also reported that cows with high weight loss postpartum had higher insulin AUC prepartum. We found a negative correlation between the expression of lipogenic genes on d −21 and plasma fatty acids on d −7 (*LPL*) and d 7 (*LPL* and *DGAT2*). This suggests that the net result of high plasma fatty acids in early lactation is due not only to insulin's loss of ability to inhibit lipolysis but rather to a broader hindering of insulin signaling causing a combination of an increase in lipolysis and a decrease in lipogenesis. Of course, the available data do not confirm the hypothesis but are indicative; more research is warranted to elucidate the relationship between IR and lipogenesis potential.

Effects of Breeding Value on Lipolysis and Lipogenesis in SAT

Breeding value for milk production was a significant factor for *LPL* and *DGAT2* mRNA abundance in SAT in the present article and for *GLUT4* mRNA abundance in SAT in the companion article (Jaakson et al., 2018). The higher the genetic merit for milk production, the lower the expression of genes responsible for bringing in (*GLUT4*, *LPL*) and accumulating energy (*DGAT2*) in SAT. Selecting animals only by their effects on milk production will increase adipose tissue mobilization in transition period, and, thus, specific traits of adipose tissue must be considered in order to increase the robustness of dairy cows.

CONCLUSIONS

High adiposity prepartum lowers the whole-body response to insulin and causes reduced expression of lipogenic genes in subcutaneous adipose tissue 3 wk before calving, but its effect was not evident postpartum. Physiological state, possibly IR, which causes more prominent insulin release after glucose infusion 3 wk before parturition, is related to higher lipid mobilization up to 2 wk postpartum and is associated with lower expression of lipogenic genes in subcutaneous adipose tissue.

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BODY CONDITION EFFECTS ON DRY MATTER INTAKE AND METABOLIC STATUS DURING THE TRANSITION PERIOD IN HOLSTEIN DAIRY COWS

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ABSTRACT. The objective was to evaluate dry matter intake, metabolite concentrations and milk production of cows with different dry period body condition score (BCS). In addition, to support these results with previously reported insulin resistance and adipose tissue mRNA data on the same cows. Multiparous Estonian Holstein cows (n = 42) were assigned to three experimental groups on the basis of BCS 28 days before expected calving (d –28) as follows: BCS ≤ 3.0 (2.25–3.00; thin (T), n = 14); BCS = 3.25–3.5 (optimal (O), n = 14); BCS ≥ 3.75 (3.75–4.50; over-conditioned (OC), n = 14). Blood samples were taken between d –21 and d 42 in relation to calving, milk production data were collected throughout lactation. The OC cows' adaptation to the demands of lactation was the worst based on the comparison of dynamics of blood parameters between BCS groups. They had the most unbalanced metabolism and used more stored lipids compared to T and O cows. Fatty acids concentrations in the first week of lactation, related to insulin resistance status in the dry period and DMI in the first days of lactation, describe most of the variation ($R^2 = 0.55$) in BCS loss during the first 42 days of lactation.

Introduction

The transition period is metabolically the most demanding period throughout the productive period, which is illustrated by high disease incidence and culling rates (Ingvartsen, Moyes, 2013). In order to have trouble-free transitions, the cow needs to successfully adapt to lactation challenges (Gross, Bruckmaier, 2019) and homeorchetic changes during pregnancy play a vital role in the ability to adapt (Bauman, Currie 1980). The main goals for herd managers during the dry-period in supporting good adaption are to avoid overfeeding, overconditioning, and declining dry matter intake (DMI) before parturition (Drackley, Cardoso, 2014).

It has been shown that overfeeding either during the far-off or close-up periods results in higher lipid mobilization postpartum (Dann *et al.*, 2006, Mann *et al.*, 2016). However, the negative effect of overfeeding seems to be more pronounced with diets high in starch and its residues as studies with grass-based feeding have not given similar results (Agenäs *et al.*, 2003, Salin *et al.*, 2018). Long term overfeeding, irrespective of diet, will lead to increased fat reserves as the excess of dietary energy is mainly stored in the adipose tissue. Body condition score (BCS) is a good proxy for the amount of fat reserves, and over-conditioned cows face a greater risk of high lipid mobilization leading to high risk for health problems, while under-conditioned cows produce less milk and have lower fertility (Roche *et al.*, 2009). The range of optimum body condition is still debated and may depend on a genotype as cows with higher genetic merit or milk production are prone to a higher rate of lipolysis compared to lesser peers (Khan *et al.*, 2013, Karis *et al.*, 2020). Adequate intake during the transition period indicates cows' adaption to lactation and leads to reduced negative energy balance (NEB). Both overfeeding and overconditioning are associated with lower DMI postpartum (Drackley, Cardoso, 2014).

At the beginning of lactation cows' endocrine status favours lipid mobilization mainly through adrenergic signalling and the blunted effect of its main antagonist insulin on adipocytes will further induce lipolysis. This

situation of insulin resistance (IR) is deemed to develop during the dry period (De Koster, Opsomer, 2013). We have previously shown that the development of IR, changes in the expression of mRNAs and proteins related to glucose and lipid metabolism are dependent on the body condition in the dry period (Jaakson *et al.*, 2018, Karis *et al.*, 2020). In this study, we set out to test the hypothesis that the factors through which the amount of fat reserves before calving affects cows' adaption to lactation are interrelated. Our objective was to evaluate DMI around calving, concentrations of metabolites related to a potential overload of metabolism, and milk production of cows with different dry period BCS.

Material and methods

Experimental design

The European Council Directive regarding the protection of animals and the Estonian Animal Protection Act have been complied with in this experiment. The study was approved by the Committee for Conducting Animal Experiments at the Estonian Ministry of Rural Affairs. The study was carried out over two consecutive years (2013–2015) on the experimental farm of the Estonian University of Life Sciences with a herd size of 120 cows and a mean annual milk yield of 9 200 kg cow^{-1}.

Cows BCS was assessed fortnightly according to the method described by Edmonson *et al.* (1989) starting from dry-off approximately 60 days before expected calving. Cows whose BCS 28 days before expected calving (d –28) was the same as at dry-off were eligible for the study. In total, 46 Estonian Holstein cows were assigned to three experimental groups on the basis of BCS on d –28 as follows: BCS ≤ 3.0 (2.25–3.00, thin (T)); BCS 3.25–3.5 (optimal (O)); BCS ≥ 3.75 (3.75–4.50, over-conditioned (OC)). Due to culling in the first two weeks of lactation, 4 cows were excluded from the study. The remaining 42 cows were equally distributed between three experimental groups, 14 each. The average parity of the cows after calving during the trial period was the following: T – 2.6 ± 0.9 (parity 2 [n = 9], 3 [n = 3], 4 [n = 1], 5 [n = 1]), O – 3.2 ± 1.2 (parity 2 [n = 5], 3 [n = 4], 4 [n = 3], 5 [n = 1], 6 [n = 1]), OC – 3.7 ± 1.0 (parity 2 [n = 1], 3 [n = 6], 4 [n = 4], 5 [n = 2], 6 [n = 1]). Cows were enrolled in accordance with blocked design with each block consisting of three cows, one from each experimental group. Fortnightly BC scoring was continued until d 42 postpartum.

On d –28 cows were removed from the dry cow barn to tie-stall housing and from the seventh milking, cows were moved to a free-stall barn with a milking parlour.

The cows were milked twice daily at 05:00 and 15:00. Milking parlour hardware (DeLaval, Tumba, Sweden) recorded milk yields. Morning and evening milk samples were collected either on Sundays and Thursdays (from March 2013 to April 2014) or on Sundays, Thursdays and Tuesdays (from May 2014 to December 2015) with in-line milk meters (DeLaval, MM27BC, Sweden). Milk samples were stabilized with bronopol (Broad Spectrum Microtabs, D&F Control Systems Inc., Norwood, MA) and were analysed for fat and protein with an automatic infrared milk analyser (System FT+, Foss Electric, Hillerød, Denmark) in the Milk Analysis Laboratory of Estonian Livestock Performance Recording Ltd. ECM-yields were calculated according to Sjaunja *et al.* (1990). A fourth-order polynomial was fitted to milk, ECM, milk fat and milk protein production values. The area under the curve was calculated for each of the variables as the definite integral of the fitted polynomial and used as an estimate for total production over 42 and 305 DIM.

Disease events during the trial period are reported elsewhere (Jaakson *et al.*, 2018), these disease events were not taken into account in the current study. In addition, three cows from group T and three cows from group OC were culled before the end of lactation. The culling reasons were the following: two incidences of feet disease and four incidences of udder disease.

We have previously reported data on glucose tolerance test carried out on d –21 and d 21, data on mRNA and protein abundance in subcutaneous adipose tissue taken on d –21 and d 21 of the same cows (Jaakson *et al.*, 2018, Karis *et al.*, 2020). Blood samples were taken approximately one hour before the start of the glucose tolerance test.

Feeding, DMI drop, BCS loss

Diets, ingredients and chemical composition are presented in table 1. Cows were fed TMR *ad libitum* twice a day, at 05:30 and 14:30. Diets were calculated according to Estonian feeding recommendations: metabolizable energy (ME) according to Oll and Tölp (1995), metabolizable protein (MP) as described by Kärt *et al.* (2002). Between d –28 to d 2 cows were fed individually and orts were collected and weighed twice daily before the fresh feed was offered. Daily intake was calculated as the difference between the weight of feed offered and the weight of orts. To calculate the DMI drop average DMI between d –14 to d –8 were calculated for each cow, which was thereafter subtracted from DMI on d –1.

Third-order polynomial was fitted to BCS data, and BCS loss was calculated as the difference between the model's value on d 42 minus the value on d 1.

Blood sampling and laboratory analyses

Blood samples were taken on d –21 ± 2.3, d –14 ± 2.1, d –7 ± 2.6, d 7 ± 0.9, d 14 ± 0.8, d 21 ± 1.0, d 28 ± 1.1 and d 42 ± 2.1 at around 10:00 from the coccygeal vein into vacuum tubes containing Li-heparin (VACUETTE®, Greiner Bio-One International GmbH, Kremsmünster, Austria). Samples were centrifuged (5 000 × g, 15 min, +4°C) and stored at –80 °C. Clinical chemistry analyser (ERBA XL300, ERBA Diagnostics Mannheim GmbH, Mannheim, Germany) was used to measure the concentration of plasma fatty acids (also known as non-esterified fatty acids (NEFA)) (cat. no. FA 115; Randox Laboratories Ltd., Crumlin, United Kingdom), β-hydroxybutyrate (BHB) (cat. no. RB 1007; Randox Laboratories Ltd.),

total antioxidant status (TAS) (cat. no. NX 2332; Randox Laboratories Ltd.), aspartate aminotransferase (AST) (product code XSYS016, ERBA Diagnostics Mannheim GmbH, Germany), glucose (product code XSYS012, ERBA Diagnostics Mannheim GmbH), albumin (product code XSYS001 ERBA Diagnostics Mannheim GmbH), blood urea nitrogen (BUN) (product code XSYS020 ERBA Diagnostics Mannheim GmbH), uric acid (product code BLT00062 ERBA Diagnostics Mannheim GmbH). All of the inter- and intra-assay CVs were below 8.3 and 6.1%, respectively.

Insulin was analysed from the same eight samples by bovine-optimized sandwich ELISA (cat no 10-1201-01; Mercodia AB, Uppsala, Sweden), with a detection limit of 0.025 ng mL^{-1}, on a microplate reader (SunriseTM, Tecan Group Ltd., Switzerland); results were calculated using cubic spline regression (Magellan™ data analysis software; Tecan Group Ltd., Switzerland). The inter-assay coefficients of variation for plasma insulin concentrations of 0.2 ng mL^{-1} and 1.6 ng mL^{-1} were 4.6 and 6.6%, respectively and the intra-assay coefficients of variation were 4.2 and 4.1%, respectively.

Statistical analysis

Statistical analyses were performed with software R (version 3.5.0, R Foundation of Statistical Computing, Vienna, Austria). The DMI data from d –28 to d –15 and from d –14 to d –8 were averaged for each cow. To estimate the effect of the BCS group and time on the dependent variables a mixed linear model was fitted, considering the fixed effects of the BCS group, time, their interaction and the confounding factors of parity and relative breeding value for milk production, and random effects of animal and block. Pre- and post-partum blood sample data were modelled separately with the exception of DMI between d –7 to d 2 in relation to calving. For variables that were measured once or pooled, the fixed effect of time and random effect of the animal was removed from the model. If the modelling resulted in a singular fit due to the effect of a block being close to zero it was removed from the model. Modelling was performed with the function "lmer" in the package "lme4" and the least square means (LSM, *alias* marginal or model-based means) were estimated with the function "emmeans". The pairwise comparison of LSM was performed with the function "contrast". P-values were adjusted for multiple testing with the Tukey method. The significance of the effect of factors considered in the model was estimated with the type 2 Wald Chi-Square Test with the function "Anova". Two analyses were performed for variables that were not normally distributed (normality tested with the Kolmogorov-Smirnov test): at first, the models were fitted and the LSM were estimated on an arithmetic scale, and subsequently, the P-values were estimated fitting the same models on logarithm-transformed values. Linear regression lines and coefficients of determination were calculated to investigate the relationships between fatty acids on d 7 and DMI on d 2, between fatty acids on d 7 and BCS loss. Statistical significance was declared at $P < 0.05$.

Table 1. Diets ingredients and chemical composition (Mean ± SD) of total mixed rations diets (published in Jaakson *et al.*, 2018 and Karis *et al.*, 2020)

Item	Diets				
	Far-off Until d –15	Close-up d –14 to –1	Lactation 1 d 1 to 6	Lactation 2 d 7 to 14	Lactation 3 from d 15
Ingredient, g kg^{-1}					
Grass silage	955 ± 81	599 ± 71	604 ± 72	460 ± 49	384 ± 37
Hay	33.6 ± 81	28.3 ± 69	28.6 ± 70	20.3 ± 49	15.6 ± 37
Barley meal	–	301 ± 10	303 ± 10	309 ± 0.1	296 ± 0.2
Cornmeal	–	–	–	64.3 ± 0.0	120 ± 0.1
Heat-treated rapeseed cake	–	47.1 ± 0.6	47.5 ± 0.6	129 ± 0.0	168 ± 0.1
Mineral-vitamin feed	11.7 ± 0.11	7.82 ± 0.21	10.5 ± 0.32	8.58 ± 0.02	7.99 ± 0.02
Anionic mineral feed[3]	–	10.4 ± 0.2	–	–	–
Limestone	–	6.26 ± 0.2	–	4.29 ± 0.0	4.41 ± 0.7
Sodium chloride	–	–	5.78 ± 0.1	4.72 ± 0.0	4.40 ± 0.0
DM of diet	348 ± 78	435 ± 64	433 ± 64	483 ± 53	511 ± 45
Chemical composition					
CP, g kg^{-1} of DM	131 ± 10.5	144 ± 7.2	145 ± 7.3	161 ± 5.6	169 ± 4.8
MP, g kg^{-1} of DM	72.8 ± 3.3	86.6 ± 2.1	87.3 ± 2.1	97.9 ± 1.6	104 ± 1.3
ME, MJ kg^{-1}	8.70 ± 0.3	10.1 ± 0.2	10.2 ± 0.2	10.9 ± 0.1	11.3 ± 0.1
NDF, g kg^{-1} of DM	534 ± 51	455 ± 32	458 ± 32	410 ± 25	380 ± 22
ADF, g kg^{-1} of DM	387 ± 47	278 ± 29	280 ± 29	242 ± 22	220 ± 18
Ca, g kg^{-1} of DM	11.4 ± 1.9	10.3 ± 1.3	9.33 ± 1.3	9.54 ± 1.0	8.97 ± 0.8
P, g kg^{-1} of DM	3.42 ± 0.4	4.06 ± 0.2	4.03 ± 0.2	4.48 ± 0.2	4.77 ± 0.2

[1] Composition (as-fed basis): 170 g kg^{-1} of Ca; 50 g kg^{-1} of P; 30 g kg^{-1} of Na; 140 g kg^{-1} of Mg; 30 g kg^{-1} of S; 1000 mg kg^{-1} of Cu; 4500 mg kg^{-1} of Zn; 4000 mg kg^{-1} of Mn; 40 mg kg^{-1} of Se; 50 mg of Co; 200 mg of I; 800 000 IU kg^{-1} of vitamin A; 190 000 IU kg^{-1} of vitamin D; and 8000 IU kg^{-1} of vitamin E.

[2] Composition (as-fed basis): 150 g kg^{-1} of Ca; 35 g kg^{-1} of P; 75 g kg^{-1} of Na; 90 g kg^{-1} of Mg; 1 g kg^{-1} of S; 4000 mg kg^{-1} of Cu; 6667 mg kg^{-1} of Zn; 6452 mg kg^{-1} of Mn; 94 mg kg^{-1} of Se; 109 mg of Co; 650 000 IU kg^{-1} of vitamin A; 150 000 IU kg^{-1} of vitamin D; and 4000 IU kg^{-1} of vitamin E.

[3] Composition (as-fed basis): 9 g kg^{-1} of Ca; 1 g kg^{-1} of P; 5 g kg^{-1} of Na; 100 g kg^{-1} of Mg; 1000 mg kg^{-1} of Cu; 5000 mg kg^{-1} of Zn; 2000 mg kg^{-1} of Mn; 27 mg kg^{-1} of Se; 40 mg of Co; 100 mg of I; 1 000 000 IU kg^{-1} of vitamin A; 60 000 IU kg^{-1} of vitamin D; and 10 000 mg kg^{-1} of vitamin E, 100 000 mcg kg^{-1} of biotin.

Results

Group differences in milk yield, DMI and BCS loss

Milk yield for the first 42 DIM, 305-day milk and ECM production did not differ between the groups, but ECM production up to d 42 was greater in group OC compared to group T ($P < 0.05$; Table 2). OC cows had a higher milk fat percentage in the first 42 days of lactation compared to O ($P < 0.05$) and T ($P < 0.01$) cows.

DMI from d –14 to d –8 was lower in OC cows compared to T cows ($P < 0.05$; Table 2). In addition, OC cows DMI were lower than those of T cows on d –3 and d –1 ($P < 0.05$; Fig. 1). On d 1 and 2 postpartum DMI was the greatest in T cows ($P < 0.01$). A DMI drop was significant for all groups (T – $P < 0.05$; O and OC – $P < 0.01$), but it did not differ between groups.

The BCS on d –28 was different between the groups ($P < 0.01$) and the loss until 42 DIM was the greatest in OC cows ($P < 0.01$).

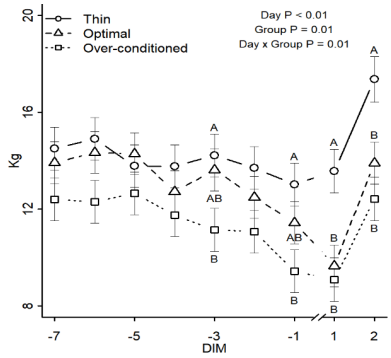

Figure 1. Dry matter intake around calving in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference ($P \leq 0.05$)

Group differences in blood metabolites

Throughout the study period OC cows had a higher concentration of fatty acids, differing from T and O cows on d –28 and d –14, and from T cows on d –7, d 7 and d 21 ($P < 0.05$; Fig. 2). The BHB concentration was lowest on d –14 for OC cows and higher than in T cows on d 14 ($P < 0.05$). On d –21 the glucose concentration in T cows was lower than in OC cows ($P < 0.05$; Fig. 3). From pre- to postpartum glucose and insulin followed the same dynamics. No differences were recorded in the concentrations of insulin between the BCS groups. The activity of AST was highest in OC cows on d 7 ($P < 0.05$), on d 14 ($P < 0.01$), and higher than in T cows on d 21 ($P < 0.05$; Fig. 4).

Albumin concentration was higher prepartum in OC cows compared to T cows ($P < 0.01$), in addition, it was higher than O cows on d –14 ($P < 0.01$; Fig. 5). No differences were recorded postpartum. No differences were also found in TAS throughout the experimental period. Uric acid concentration differed only on d –21, at which point OC cows had greater concentrations compared to T cows (Fig. 6). The BUN concentration did not differ between groups prepartum, but in the postpartum period, T cows had the highest concentration on d 21 (compared to O, $P < 0.05$; compared to OC, ($P < 0.01$)) and higher than OC cows on d 28 ($P < 0.05$).

Associations between DMI, BCS loss and blood fatty acids

In the calculation of regression lines and coefficients of determination, the BCS groups were not differentiated. There was a negative correlation between fatty acids on d 7 and DMI on d 2 and a positive correlation between fatty acids on d 7 and BCS loss during the first 42 DIM (Fig. 7). DMI on d 2 described 37% of fatty acids variance on d 7 and fatty acids on d 7 described 55% of BCS loss variance ($P < 0.01$).

Table 2. Milk yield and composition, DMI, and body condition score (BCS) characteristics. Values are expressed as LSM. Letters "a", "b" and "c" indicate a difference ($P \leq 0.05$)

Characteristic	Group			SEM	P-value
	Thin	Optimal	Over-conditioned		
Milk					
up to d 42, kg d^{-1}	36.0	36.7	38.0	54.4	0.573
up to d 305, kg	10 079	10 639	10 824	463	0.495
ECM[1] up to d 42, kg d^{-1}	37.1^{a}	38.6^{ab}	42.7^{b}	56.2	0.019
ECM up to d 305, kg	9 901	10 484	10 909	384	0.133
Fat up to d 42, %	4.25^{a}	4.38^{a}	4.99^{b}	0.15	0.000
Fat up to d 305, %	3.89	3.87	4.02	0.14	0.717
Protein up to d 42, %	3.32	3.36	3.33	0.06	0.816
Protein up to d 305, %	3.22	3.31	3.34	0.06	0.308
DMI, kg					
d –28 to d –15	14.2	13.8	12.2	0.72	0.126
d –14 to d –8	15.1^{a}	14.8^{ab}	12.7^{b}	0.68	0.017
drop[2]	1.77	3.52	3.46	0.85	0.235
BCS					
on d –28	2.86^{a}	3.33^{b}	3.89^{c}	0.06	0.000
loss[3]	0.45^{a}	0.64^{a}	1.07^{b}	0.08	0.000

[1] Energy corrected milk, calculated according to Sjaunja *et al.*, 1990.
[2] The subtract between the average DMI on d –14 to d –8 and d –1.
[3] Total BCS loss on the first 42 days in milk.

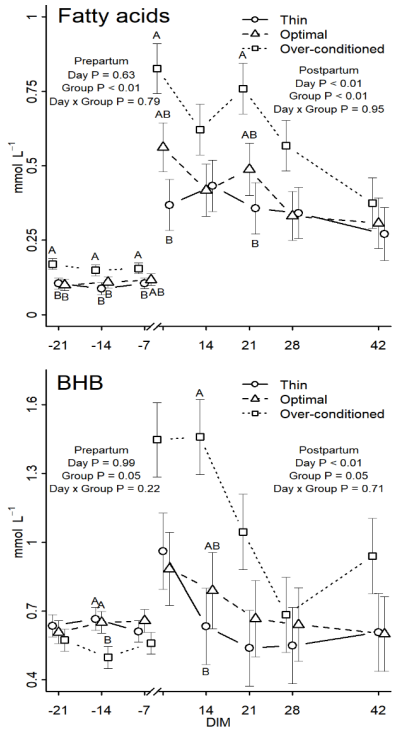

Figure 2. Concentrations of fatty acids and β-hydroxybutyrate (BHB) during the experimental period in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference (P ≤ 0.05)

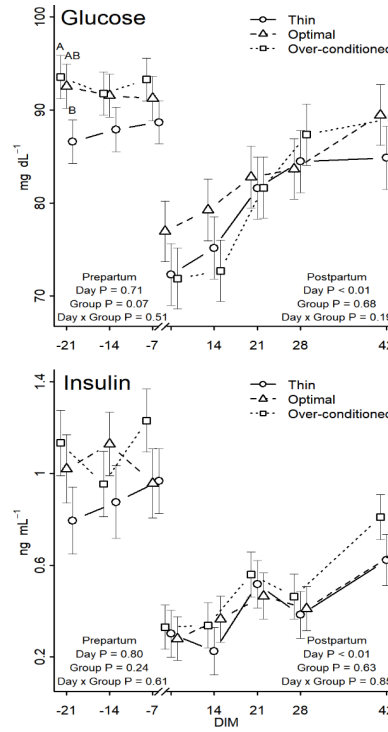

Figure 3. Concentrations of glucose and insulin during the experimental period in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference (P ≤ 0.05)

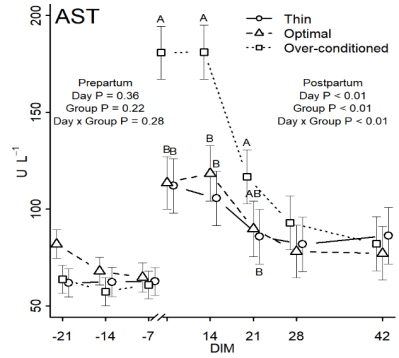

Figure 4. The activity of aspartame aminotransferase (AST) in plasma during the experimental period in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference (P ≤ 0.05)

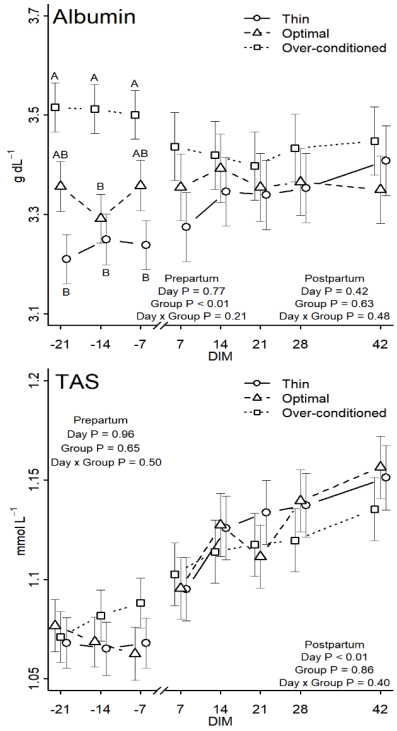

Figure 5. The concentration of albumin and total antioxidant status (TAS) during the experimental period in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference (P ≤ 0.05)

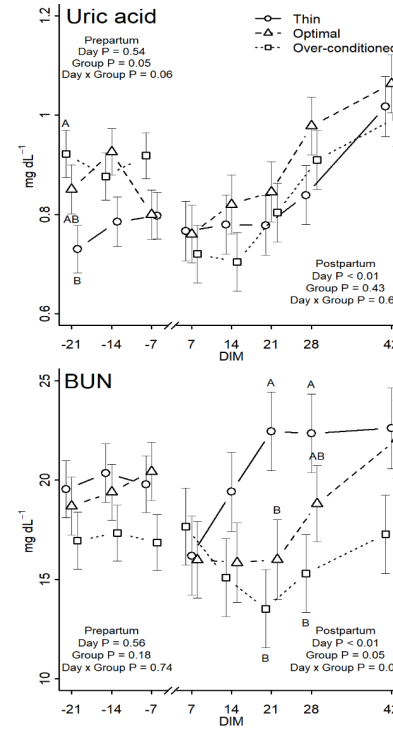

Figure 6. The concentrations of uric acid and blood urea nitrogen (BUN) during the experimental period in multiparous Holstein cows grouped according to BCS on d –28 as follows: ≤ 3.0 – Thin; 3.25–3.5 – Optimal; ≥ 3.75 – Over-conditioned (n = 14 each). Values are expressed as LSM ± SEM. Letters "A" and "B" indicate a difference (P ≤ 0.05)

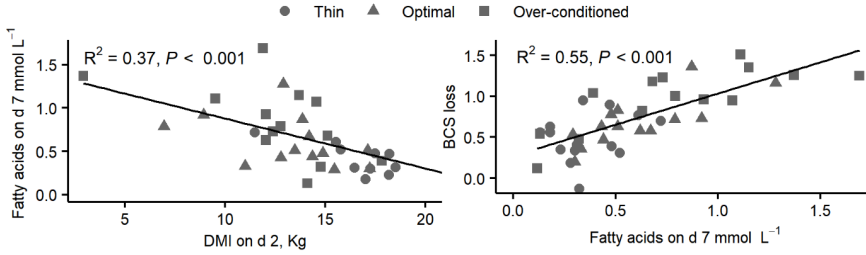

Figure 7. Regression line and coefficient of determination (R^2) of dry matter intake (DMI) on d 2 postpartum and body condition score (BCS) loss during first 42 days in milk with plasma fatty acids concentration on day 7. Points represent the values of individual multiparous Holstein cows

Discussion

This paper is a companion paper to that of Jaakson *et al.* (2018) and Karis *et al.* (2020) and proposes an integration of those findings with longitudinal dynamics in DMI, BCS, blood metabolite concentrations and milk composition of the same cows.

A limitation of our study is the uneven distribution of parity between the experimental groups. To compensate, we added the effect of parity to each mixed linear model as a confounding factor. In most cases, the effect

of parity was small and insignificant; the only exception is DMI between d –28 to d –15.

The magnitude of change in most of the concentrations of the measured metabolites illustrates the drastic shift in cows' metabolism in order to adapt to lactation.

The effect of BCS on DMI, milk and blood biomarkers

Greater glucose concentrations on d –21 and fatty acids concentrations throughout the prepartum period in OC cows compared to T cows might be a reflection of IR, as we have previously reported that OC cows had greater glucose and insulin AUC (Jaakson *et al.*, 2018) as well as longer fatty acids latency (Karis *et al.*, 2020) after glucose infusion on d –21. The differences in glucose concentrations between groups disappeared after d –21 probably because the DMI of OC cows was lower between d –14 to d –8 compared to T cows, and diet starch concentrations were increased two weeks before calving.

We observed differences in uric acid and albumin concentrations prepartum, but the physiological causes and meaning remain uncertain. Albumin serves as a marker of liver activity, inflammation, protein degradation or undernutrition (Agneäs *et al.*, 2003; Bertoni, Trevisi, 2013), but neither of those seems plausible explanations for these cows prepartum. Roche *et al.* (2013) also found a tendency for lower albumin in thin cows prepartum. The difference in uric acid, a marker for microbial protein degradation in the intestine that correlates with DMI (Tas, Susenbeth, 2007), is counter-intuitive as its concentration was lower for T cows even though the DMI was higher on d –28 to d –15 (though, no significance was observed).

It has become common knowledge that a dairy cow's DMI gradually decreases up to 30% over the last three weeks of gestation (Ingvartsen, Andersen, 2000). However, our findings do not support this notion as we observed a DMI drop only in the final days of gestation, and this seemed to be dependent on BCS as we saw a slight DMI drop in T, but a noticeable drop in O and OC cows in the final days of gestation. In agreement with our results, Agenäs *et al.* (2003) and Salin *et al.* (2018) did not report a depressed DMI during close-up in cows overfed with grass silage. High starch intake seems to be one of the possible reasons for DMI depression prepartum (Grummer *et al.*, 2004, Roche *et al.*, 2013). The reason could be the fact that glucose, availability of which is increased, is more tightly regulated and has more of an effect on the endocrine system (Bradford, Allen, 2007). We hypothesize that grass-based diets in the dry period protect cows from a gradual DMI drop. This is also consistent with the hepatic oxidation theory for DMI as acetate, the more dominant volatile FA in grass silage based diets is not extracted from the blood by the liver (Allen, 2020).

The DMI drop for O and OC cows resulted in a difference between T cows in the first two days of lactation. Regarding the T and O groups, this difference was not reflected in the concentrations of blood metabolites as we only recorded differences on BUN concentrations on d 21 between the groups postpartum. Our DMI data end at d 2, thus there is a 5-day discrepancy between the first postpartum blood samples. We hypothesize that the DMI of O cows must have caught up with that of the T cows. This is supported by the facts that we previously reported no difference in the total energy balance during the first 21 DIM (Jaakson *et al.*, 2018) for the same cows nor a difference in body condition loss between groups T and O.

Even though OC cows' fatty acids and BHB concentrations postpartum differed only from the T cows, we conclude that OC cows had the highest lipolysis up to the third week of lactation. We argue that the excess of fatty acids released from the adipose tissue of OC cows was divided between compartments within the organism (*e.g.* liver, udder) and synthesized into other metabolites (*e.g.* BHB), thereby lowering fatty acids concentration in plasma. This is supported by the very high BHB concentrations on d 7 and d 14 in OC cows and the highest total ECM production during the first 42 DIM, which is driven mainly by a high-fat content in milk. In the first week of lactation, the uptake of fatty acids from the blood by the mammary gland accounts for approximately 40% of total milk fatty acids (Bell, 1995). Probably the OC cows' high milk fat percentage, particularly in the first week of lactation, is caused by high lipolysis and fatty acids concentrations in the blood. This is supported by the highest C18:1 *cis*-9 fatty acid concentration in the milk ($P < 0.01$, unpublished data) and the greatest BCS loss in OC cows during six weeks of lactation.

The high activity of AST in OC cows agrees with other metabolites and milk traits data indicating again high lipolysis in OC cows that put them at risk of metabolic and infectious disease (Ingvartsen, Moyes, 2013). Over-conditioned cows are known to be at risk for fat infiltration to liver cell and AST activity is one of its indicators (Bobe *et al.*, 2004). In agreement with his, low urea is also associated with increased TAG in the liver (Jorritsma *et al.*, 2001) and lower expression of some mRNA encoding of enzymes involved in ureagenesis in the liver for cows at risk of high lipid mobilization (Graber *et al.*, 2010). Pires *et al.* (2013) reported evidence indicating higher labile protein mobilization in thin cows, presumably to compensate for the lack of energy from adipose tissue, thus it is plausible that the highest BUN for T cows on d 21 may arise from the differences in protein degradation in addition to liver function.

Increasing TAS postpartum shows that dairy cows are challenged with high rates of oxidation, which is caused by ongoing metabolic stress and NEB. This leads to increased synthesis of reactive oxygen species that may overwhelm the antioxidant capacity and lead to oxidative stress and inflammation response (Sordillo, Raphael, 2013). Even though high lipid mobilization (Sordillo, Raphael, 2013) and high BCS (Bernabucci *et al.*, 2005) are associated with systemic oxidative stress and inflammation, we observed no

differences in TAS or albumin between the BCS groups postpartum. There are more specific biomarkers for inflammation and oxidative stress, for example, tumour necrosis factor α and glutathione peroxidase, than used in this study and tissue-specific alterations might occur (*e.g.* adipose tissue inflammation) (Contreras *et al.*, 2018), therefore a definitive decision on this cannot be made.

Association between DMI, fatty acids and BCS loss

DMI on d 2 describes 37 per cent of the variation of fatty acids on d 7, which in turn seems to be the major determinant for the overall BC loss over the first 6 weeks of lactation. Thus, the success of the transition period is determined in early lactation and relies on the adaption of cows metabolism to a new physiological state during the dry period. In agreement, according to the hepatic oxidation theory, increased plasma fatty acid concentration, and thus intensified oxidation in the liver, is the limiting factor for DMI during early postpartum (Allen, Piantoni, 2013) and improving cows DMI intake pre- and postpartum is seen as the main goal to ensure a trouble-free transition period (Drackley, Cardoso, 2014). De Koster *et al.* (2019) clustered cows based on glucose, fatty acids, BHB and insulin-like growth factor 1 concentrations in blood at the beginning of lactation and reported that the main differences between metabolically balanced and unbalanced cows are DMI and BCS loss postpartum. Our data show that in addition to unfavourable metabolite concentrations OC cows had lower DMI compared to T cows and greater BCS loss than T and O cows and therefore can be classified as metabolically unbalanced. Although, DMI between OC and O cows did not differ on d 1 and 2 postpartum, OC cows still mobilize more body lipids, especially in the first weeks of lactation, suggesting that there are more factors determining the balance of metabolism or, in other words, OC cows experience higher lipolysis regardless of DMI. As only two days of postpartum DIM data was available in our study, and it might not reflect the actual feed intake during early lactation, our interpretation must be taken with caution. The underlying reason for higher lipolysis might be IR, as we have shown its interaction with body condition, and that cows with greater insulin AUC prepartum have decreased lipogenesis potential (lower *LPL* and *DGAT2* mRNA abundance) and greater fatty acids concentration on d 7 (Karis *et al.*, 2020). With good management practices the BCS of dry off cows can be optimized, but the reason for IR prepartum needs to be further studied, including the role of a genetic component in its development.

Conclusions

Overconditioning causes minor differences in glucose and fatty acids concentrations during the last three weeks of prepartum. During the first six postpartum weeks, the OC cows' adaptation to the demands of lactation was the worst, they had the most unbalanced metabolism and used more stored lipids compared to T and O cows. This also reflected in higher milk fat percentage and higher ECM production in OC cows during the first six lactation weeks. Fatty acids concentrations in the first week of lactation, related to IR status in the dry period and DMI in the first days of lactation, describe most of the variation in BCS loss during the first six weeks postpartum. Thus, metabolic processes during the dry period and in the first week of lactation are important determinants for metabolic health in the first weeks of lactation.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

HJ, KL, MH, AW, MO – study conception and design;
PK, HJ, KL, MH, AW – acquisition of data;
PK, HJ, KL – analysis and interpretation of data;
PK – drafting of the manuscript;
PK, HJ, KL, MR, MH, AW, MO – critical revision and approval of the final manuscript.

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2017	"DK.5 Molecular Biology in Animal Sciences." International Winter School of Graduate School in Biomedicine and Doctoral School of Clinical Medicine, Tartu, Eesti. 2 EAP.
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2018	"Matemaatiline statistika, statistiline modelleerimine". Eesti Maaülikool, Tartu, Eesti. 1 EAP.
2019	"Biomarkers, sensors, physiology." Eesti Maaülikool, Tartu, Eesti. 3 EAP.
2021	"Feed Ration Planning in Dairy Cattle Herds." Aarhus University, Foulum, Taani. 5 EAP.

LIST OF PUBLICATIONS

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3.4. Articles/presentations published in conference proceedings

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VIIS VIIMAST KAITSMIST

IMBI NURMOJA
SIGADE AAFRIKA KATKU EPIDEMIOLOOGIA EESTIS JA ÜHE VIIRUSTÜVE ISELOOMUSTUS
EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN ESTONIA AND CHARACTERIZATION OF ONE VIRUS STRAIN
Doktor **Arvo Viltrop**, doktor **Sandra Blome**, doktor **Klaus Robert Depner**
15. juuni 2021

MERILI TOOM
ERINEVATE VAHEKULTUURIDE BIOMASSI MOODUSTAMISE JA LÄMMASTIKU SIDUMISE VÕIME SÕLTUVALT KÜLVIAJAST NING VAHEKULTUURIDE MÕJU SUVIODRA SAAGILE
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16.juuni 2021

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